FETAL-PLACENTAL CALCIUM METABOLISM IN MICE PARTLY OR FULLY DEFICIENT IN PARATHYROID HORMONE

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by

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ABSTRACT

It is well established that parathyroid hormone (PTH) plays an essential role in regulating calcium and bone homeostasis in the adult. The role, if any, of PTH in fetal-placental mineral homeostasis has been uncertain. Therefore, the purpose of the present doctoral research was to examine the role of PTH in fetal-placental calcium homeostasis. It was hypothesized that: *PTH, despite its low circulating levels during fetal life, plays an important role in regulating not only fetal blood calcium and skeletal development, but also placental calcium transfer.* To address this, two different genetic mouse models of PTH deficiency were utilized. The *Pth*^{tm1Dgo} knockout (i.e. *Pth* null) mice served as a model of complete absence of PTH because they have enlarged parathyroids that are incapable of making PTH, while the *Gcm2*^{tm1Kry} knockout (i.e. *Gcm2* null) mice served as a model of severe hypoparathyroidism because they lack parathyroids but have some PTH.

Both nulls displayed a fetal hypoparathyroid phenotype, experiencing hypocalcemia, hypomagnesemia, hyperphosphatemia, low amniotic fluid mineral content, and reduced skeletal mineral content. When *Pth* null fetuses were treated *in utero* with PTH (1-84), placental calcium transfer increased, and placental gene expression was altered. It was also discovered that PTH is expressed in the placenta of wild-type and *Gcm2* null fetuses. Thus, from the present study it is evident that PTH does indeed play an important role in fetal-placental mineral homeostasis. More specifically, PTH is important for fetal blood calcium, fetal skeletal development, is expressed locally

in the placenta, regulates placental gene expression, and may directly regulate the transfer of calcium from mother to fetus.

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LIST OF ABBREVIATIONS

% of +/- Mean	Percent of Heterozygous Mean
+/- or HET	Heterozygous
$1,25(OH)_2D_3$	1,25- Dihydroxyvitamin D_3 (i.e. active form of vitamin D)
1α(OH)ase	1-alpha-hydroxylase
AC	Adenylyl Cyclase
ANOVA	Analysis of Variance
bp	Base Pair
C-section	Cesarean section
Ca	Calcium
Ca^{2+}ATPase	Calcium Adenosinetriphosphatase
CaBP9k	Calbindin-D9K (i.e. S100g)
cAMP	Cyclic Adenosine Monophosphate
CaSR	Calcium-Sensing Receptor
cDNA	Complementary DNA
Chga	Chromogranin A gene
C-PTHR	Carboxy-terminal PTH Receptor
C_T	Threshold Cycle
C-terminal	Carboxy-terminal (i.e. COOH-terminal)
CCAC	Canadian Council on Animal Care
CgA	Chromogranin A

Cr	Chromium
DAG	Diacylglycerol
DNA	Deoxyribonucleic Acid
dATP	Deoxyadenosine Triphosphate
dCTP	Deoxycytidine Triphosphate
dGTP	Deoxyguanosine Triphosphate
dNTP's	Deoxynucleotide Triphosphates
dTTP	Deoxythymidine Triphosphate
ED	Embryonic Day
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-Linked ImmunoSorbent Assay
EtOH	Ethanol
FDR	False Discovery Rate
Gc	Group-specific Component (i.e. Vitamin D-binding Protein)
Gcm2	Glial cells missing2
HCL	Hydrogen chloride
Hoxa3	Homeobox A3
IACC	Institutional Animal Care Committee
IP_3	Inositol Triphosphate
IPYS	Intraplacental Yolk Sac
KCL	Potassium Chloride

KH_2PO_4	Potassium Dihydrogen Phosphate
KOH	Potassium Hydroxide
L	Liter
LPE	Local-Pooled-Error
M	Molar
$MgCl_2$	Magnesium Chloride
µCi	Microcurie
µg	Microgram
µl	Microliter
ml	Milliliter
mM	Millimolar
mmol	Millimoles
mRNA	Messenger Ribonucleic Acid
n	Number of Observations
NaCl	Sodium Chloride
Na_2HPO_4	Disodium Phosphate (Anhydrous)
NaOAc	Sodium Acetate
NEO	Neomycin
NHERF1	Sodium (Na+)/Hydrogen (H+) Exchanger Regulatory Factor 1
NHERF2	Sodium (Na+)/Hydrogen (H+) Exchanger Regulatory Factor 2

nmol	Nanomole
NS	Not-Significant
OH	Hydroxide
OPG	Osteoprotegerin
p	Probability
PBS	Phosphate Buffered Saline
PCR	Conventional Polymerase Chain Reaction
Pg	Picogram
PHF	Parathyroid Hypertensive Factor
PKA	Protein Kinase A
PKC	Protein Kinase C
PLC	Phospholipase C
pM	Picomolar
PO_4	Phosphate
PTH	Parathyroid Hormone
PTH1R	Type 1 PTH/PTHrP Receptor
PTH2R	Parathyroid Hormone 2 Receptor
PTHrP	Parathyroid Hormone-Related Protein
RAM	Robust Multi-array Average
RANK	Receptor Activator of Nuclear factor kappa B
RANKL	RANK Ligand

Real-Time Quantitative RT-PCR	Real-Time Quantitative Reverse Transcriptase-PCR
RIA	Radioimmunoassay
RIN	RNA Integrity Number
RNA	Ribonucleic Acid
rpm	Revolutions Per Minute
SDS	Sodium Dodecyl Sulfate
SE	Standard Error
TE	Tris EDTA Buffer
TIP39	Tuberoinfundibular Peptide of 39 Residues
TIU	Trypsin Inhibitor Units
TRPV6	Transient Receptor Potential Cation Channel, Subfamily V, Member 6
VDR	Vitamin D Receptor
WT	Wild-type

LIST OF APPENDICES

I. Introduction

A. Preamble

By examining fetal mice deficient in parathyroid hormone (PTH) (*Gcm2* null mice) and completely lacking PTH (*Pth* null mice), the present work provides some clarity on the role of PTH in fetal calcium and bone metabolism, and some very exciting new evidence to support a role for PTH in regulating the transfer of calcium across the placenta from mother-to-fetus. The role of PTH in fetal life has been uncertain for quite some time, and it has not been thought to have a role in regulating placental calcium transfer. To gain a better understanding of our prior knowledge about the role of PTH in regulating fetal-placental calcium and bone metabolism, this review will start by summarizing how calcium metabolism is regulated in the adult, and then how the fetal system differs. Next, the expression, signaling, and receptors for two very important calcitropic hormones, PTH and parathyroid hormone-related protein (PTHrP), will be reviewed. Finally, previous studies will be reviewed which examined the role of PTH in regulating fetal blood calcium, fetal skeletal development and mineralization, and placental calcium transfer.

B. Adult Calcium Homeostasis

The adult skeleton has evolved to serve several functions including structural support, movement, protection of vital organs, and maintenance of calcium homeostasis (1). In the adult human, there is approximately 1000g of calcium and 99% of this is stored in the skeleton as hydroxyapatite crystals [Calcium $(Ca)_{10}$ Phosphate $(PO_4)_6$

hydroxide $(OH)_2$]. The remaining 1% of calcium in the body can be found in the blood, extracellular fluid and soft tissues. In the blood, 50% of the calcium is ionized and can move into cells and activate cellular processes. Thus, this fraction is known as the biologically active portion of calcium and in humans it is typically maintained at a concentration of 1.15 – 1.30 mmol/L, while the total serum concentration is typically 2.20 – 2.60 mmol/L. The remaining 50% of calcium has no function except to serve as a store of calcium, with 40% circulating bound in a pH-dependent manner to proteins (mainly albumin) and 10% circulating complexed to either phosphate or citrate. The concentration of ionized calcium in the blood is tightly regulated. It is required for numerous important biological processes including muscle contraction, neuronal excitability and plasma membrane integrity, just to name a few (2, 3). One of the central players in maintaining calcium homeostasis is PTH. Its actions on the skeleton, intestine, and kidneys serve to maintain the ionized calcium and the overall balance of calcium in the body (4).

Calcium-sensing receptors (CaSR's) are G protein-coupled receptors that are located on the plasma membrane of parathyroid cells (5). They act as a calcium thermostat, or "calciostat", that detects fluctuations of calcium in the blood. Activation of the CaSR by calcium binding leads to inhibition of PTH synthesis and release. When ionized calcium falls below the target level, the inhibitory signaling by the CaSR is removed, and PTH is released (4, 6). PTH works in several classical ways to return the ionized calcium level to normal. In bone, calcium is initially immediately released from a pool of calcium that is stored near the bone surface. If the blood calcium remains low for

several hours, calcium is also then released from an addition pool of calcium that has a slower turnover (4). However, with extended periods of hypocalcemia, PTH acts to increase osteoclastic bone resorption to liberate calcium (and phosphorous) from the skeletal reservoir (6). This is accomplished through the receptor activator of nuclear factor kappa B (RANK)/RANK ligand (RANKL)/osteoprotegerin (OPG) triad. In this system, when PTH binds to the type 1 PTH/PTHrP receptor (PTH1R) located on osteoblasts (bone forming cells), osteoblastic bone formation is initially stimulated. With prolonged stimulus by PTH, the expression of RANKL (on the surface of osteoblasts) and macrophage-colony stimulating factor (M-CSF) is increased. As a result of these stimuli, osteoclast precursors differentiate into mature osteoclasts (bone resorbing cells), and the activity of existing mature osteoclasts is increased. The prolonged binding of PTH to the PTH1R on osteoblasts also results in a decrease in the expression of OPG, which is a soluble receptor expressed by osteoblasts, that is often referred to as a "decoy" receptor because of it homology to RANK. The decrease in OPG and the increase in RANKL and M-CSF together stimulate the activity of mature osteoclasts and osteoclastogenesis, causing both calcium (and phosphorous) to be released into the blood to help return the blood calcium level to normal (4) (**Figure 1**).

In the kidney, PTH has little effect on the proximal tubule where 65% of filtered calcium is normally reabsorbed. However, in both the cortical thick ascending limb of the loop of Henle (where 20% of filtered calcium is normally absorbed), and the distal convoluted tubule (where 15% of calcium is normally reabsorbed), PTH immediately stimulates calcium reabsorption and inhibits phosphate reabsorption. Thus, under the

influence of PTH, less calcium but more phosphate is excreted in the urine; this helps increase the level of calcium in the blood (3, 4). With prolonged PTH binding to the PTH1R in the kidney, the activity of 1-alpha-hydroxylase (1α(OH)ase) is stimulated, which leads to an increase in the conversion of circulating 25-hydroxyvitamin D_3 to the active 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$). This increase in $1,25(OH)_2D_3$ in turn enhances intestinal calcium absorption which also helps to increases the blood calcium level (3, 4).

In summary, when the blood calcium level decreases below normal, the CaSR's on parathyroid glands are not stimulated and PTH is released into the circulation. PTH then acts directly in bone to increase bone resorption, and in kidney to increase calcium reabsorption, and indirectly in the intestines to enhance calcium absorption. Overall, the net effect of these actions results in the blood calcium level being restored to normal (3, 4) (**Figure 2**). The opposite is also true: when blood calcium rises above the normal range, activation of the CaSR's on parathyroid cells inhibits PTH release and less $1,25(OH)_2D_3$ is produced by the kidneys. As a result, less calcium is released from the skeleton, less calcium is reabsorbed by the kidneys, and intestinal calcium absorption is decreased. Thus, the overall effect is that blood calcium level decreases back to the normal level (3, 4).

C. Fetal Calcium Homeostasis

Although it is known that calcium and bone metabolism is regulated differently in the fetus as compared to the adult, relatively little is known about how the fetus maintains

mineral homeostasis. This is largely in part due to the understandable limitations of studying human fetuses. Thus, much of what we do know regarding fetal regulation of calcium homeostasis has been inferred from animal studies. For numerous reasons however, including the fact that much of the data are consistent among mammalian studies and that placentation is very similar between humans and rodents, it is likely that such findings also apply to humans. The possibility exists that species differences occur between animals and humans, but the information gathered from animal research is extremely critical because equivalent human data will never be possible.

The fetal period is unique. It is a time of rapid development and specific needs are placed upon the developing fetus. First, the fetus must meet the demand to provide the required amount of calcium and other minerals to fully mineralize the fetal skeleton before birth. One might anticipate that this process occurs slowly throughout gestation, however, mineralization of the fetal skeleton occurs quite rapidly in the latter part of pregnancy; in humans 80% of the 20-30g of mineral required to mineralize the fetal skeleton before birth is accreted in the third trimester (7), while rats accrete 95% of the required 12.5 mg during the last 5 days of a 3 week gestational period (8). Second, the fetus must maintain physiologically appropriate levels of calcium and other minerals that are needed for the fetal system to function normally. More specifically, in several species including humans (9, 10), sheep (11), cattle (12) and rodents (11), the fetal blood calcium is normally maintained at a higher level than that of the mother. This increase in blood calcium is mainly due to an increase in the ionized calcium level, although total calcium is also elevated (13). The physiological relevance of this fetal hypercalcemic state

remains unknown. However, it appears to exist independently of the maternal calcium level, because the fetal blood calcium level is usually unaltered in situations where the mother experiences either hypocalcemia (14) or hypercalcemia (15).

Studies in humans (10, 16), rats (17, 18), and sheep (19), have shown that fetal serum phosphorous levels are also maintained at a higher level than that observed in the mother. Like calcium, it is likely that the high levels of phosphorous in the fetus facilitate normal bone formation.

Studies comparing fetal serum magnesium levels to maternal levels have shown varying results between species and studies. However, the majority of studies conclude that fetal magnesium is approximately equal to the maternal level, or at most, modestly increased or decreased (20-23).

In the fetal milieu, many calcitropic hormones including PTH, $1,25(OH)_2D_3$, and PTHrP are also maintained at levels that differ from the adult. In many species, including humans (24), lambs (25), cattle (12), and rodents (unpublished data), fetal full length or intact PTH (i.e. PTH [1-84]) levels are significantly lower than maternal levels. This low level of PTH in the fetus has been noted in late gestation, however it is still unclear whether this low level is maintained throughout gestation, after the parathyroids form and start to produce PTH on embryonic day 11.5 (26) , or if it is just suppressed late in the gestational period (20). Several studies in rats and non-human primates have shown that PTH does not cross the placenta (18, 27, 28). Consistent with this, fetal mice lacking parathyroids had undetectable levels of PTH despite having mothers with near normal PTH levels (20). Thus, it is likely that the PTH detected in the fetal circulation derives

solely from fetal tissues. Although PTH levels are very low in the fetal circulation, it still appears to be biologically important because fetal mice that lack parathyroids are hypocalcemic and have undermineralized skeletons (29).

Like PTH, $1,25(OH)_2D_3$ also circulates at lower levels in the fetus as compared to the mother (20, 30). Furthermore, $1,25(OH)_2D_3$ detected in the fetal circulation is also considered to be mainly, if not completely, derived from fetal tissue because it is also thought to not cross the placenta (20, 31, 32). Unlike PTH however, $1,25(OH)_2D_3$ may not play a critical role in fetal mineral homeostasis. This is because no impairments in skeletal mineralization or serum mineral concentrations were noted in vitamin D receptor (*Vdr*) null fetal mice as compared to their WT littermates (33).

PTHrP is also maintained at a different level in the fetus as compared to the adult, with PTHrP circulating at higher levels in the fetus. It is normally not detected in the adult circulation, except during pregnancy and lactation (34). However, even at term, studies have reported that PTHrP levels in human umbilical cord blood are significantly higher than that of simultaneous maternal PTHrP levels (35, 36). Furthermore, when expressed in molar units and compared to simultaneous PTH levels in cord blood at term, levels of PTHrP are approximately 15-fold higher than PTH (32). Given that the molecular weight of intact PTHrP is twice that of PTH, and that PTH cannot cross the placenta, it is also unlikely that PTHrP would cross the placenta. Thus, similar to both PTH and $1,25(OH)_2D_3$, whole length PTHrP circulating in the fetus is believed to be exclusively derived from fetal tissues. However, although it has not been studied, it

remains possible that smaller fragments of PTHrP could cross the placenta, and that some might have biological activity (20, 31).

Although the fetus differs from the adult with respect to the level of minerals and calcitropic hormones that it maintains in the blood, like the adult, it can also utilize the kidneys, intestine and bone to maintain the appropriate fetal blood calcium level. Calcium, phosphorous and magnesium that is filtered by the fetal kidneys can be partly reabsorbed into the circulation to maintain the blood calcium concentration. However, the precise handling of calcium, and other minerals by the kidneys may not be as important in the fetus as in the adult. This conclusion is based on several factors. First, it has been suggested that the response to PTH in the fetal kidneys is blunted compared to the adult (37, 38). This blunted response has been associated with very low expression levels of the CaSR in the fetal kidneys, which increases substantially over the first postnatal week (39), along with the renal tubular response to PTH (37). Second, minerals which are excreted in the urine by the fetal kidneys are not permanently lost. Fetal urine, containing the non-reabsorbed minerals, is excreted into the amniotic fluid, which may then be swallowed by the fetus, allowing the minerals to be absorbed by the intestine. Similar to the adult, maintaining the appropriate level of calcium in the blood is of vital importance. Thus, as in the adult, calcium from the developing skeleton can be reabsorbed to supply calcium to the blood if necessary (20, 32, 34).

In addition to the kidneys, intestine and bone, the fetus has another organ which plays a vital role in the overall regulation of calcium homeostasis. The placenta is of vital importance for the fetus because it is responsible for, among many other things,

transferring calcium (and other minerals) from the mother to the fetus (20, 32, 40). This transfer can occur via simple diffusion, paracellular transport, vesicular transport, or by active transport (20). One may think that the transfer of calcium from the mother to the fetus is a one-way process. However, several studies have shown that there is some degree of backflux. That is, some calcium from the fetal circulation returns to the maternal circulation. This fetal-to-maternal backflux is difficult to measure accurately. Some studies have reported that the backflux is less than 1% of the forward (maternal-to-fetal) flow (41, 42) while others reported that it may be as high as 80% of the forward flow (41). The reason for these vast differences is unknown, however, it has been suggested that the differences may reflect true differences between the species that have been studied, or differences in the methodology that was used to assess backflux in the various studies (20).

The role of the placenta will be discussed in greater detail in section I.

D. Parathyroid Hormone (PTH)

PTH is an 84-amino acid single-chain polypeptide (43). In humans, the PTH gene is located on the short arm of chromosome 11 (44), while in mice it is located on chromosome 7 (45). The transcription product encodes both a "pre" signal sequence of 25 amino acids and a "pro" sequence of 6 amino acids, in addition to the mature 84-amino acid peptide. As a result, the initial translational product is a 115-amino acid product called preproparathyroid hormone (pre-pro-PTH). The pre-pro sequence is very important as it ensures proper PTH intracellular transport and processing. If it is mutated or absent,

hypoparathyroidism may occur (43). Once the pre-pro signaling sequence has been cleaved, the resulting mature PTH [1-84] peptide is then packaged into secretory/storage granules within the parathyroid cells. The granules which store and secrete PTH can either contain only the full-length 84-amino acid PTH, or PTH together with the proteases cathepsins B and H. Granules that do not contain the proteases secrete only intact PTH. Granules which contain the proteases contain a mixture of amino-terminal and C-terminal hormone fragments of PTH (46).

As previously discussed, PTH secretion is directly responsive to the ionized calcium concentration. In response to low blood calcium, PTH from the parathyroid glands is released into the circulation and returns the blood calcium to normal through its actions on bone and kidney. The concentration of calcium found in the blood has also been shown to regulate the abundance of various PTH derived peptides that are released into the circulation. Thus, when serum calcium is low, more intact PTH [1-84] is detected in the circulation. When the blood calcium is high, more C-terminal fragments such as PTH [37-84] are found (46).

1. PTH Receptors and Signaling

Type 1 PTH/PTHrP Receptor (PTH1R)

The PTH1R is highly expressed in both bone and kidney where it is the target for the classical actions of PTH to regulate calcium homeostasis. It is also expressed in many other adult tissues including the aorta, brain, breast, heart, skeletal muscle, skin and the

placenta (just to name a few), which are sites that are not normally thought of as being targets for PTH action (47).

The PTH1R appears to be the major receptor mediating the classical actions of PTH. These classical actions occur when the amino-terminal region of PTH (PTH [1-34]) which has strong homology among mammalian species (43), binds to the PTH1R and activates several signaling pathways. More specifically, upon receptor activation, a cascade of events occurs which is mediated by the PTH1R coupling to different G proteins. Via $G_{\alpha s}$, adenylyl cyclase (AC) is stimulated. This results in the formation of cyclic adenosine monophosphate (cAMP) which activates protein kinase A (PKA). However, when PTH1R activation results in $G_{\alpha q}$ activation, phospholipase C (PLC) signaling occurs. Subsequently, diacylglycerol (DAG) and inositol 1, 4, 5-triphosphates (IP_3) is formed resulting in protein kinase C (PKC) activation and the release of intracellular calcium. The end result of both AC and PLC pathway activation is that downstream biological responses are stimulated. However, whether the AC or PLC signaling pathway is activated, is tissue/cell specific. That is, in some tissues/cells the AC pathway will be activated, while in others the PLC signaling cascade will occur. This cell-specific receptor signaling may be explained by the sodium (Na^+) - hydrogen (H^+) exchanger regulatory factor 1 and 2 (NHERF1 and NHERF2), because it has been shown to be capable of changing PTH1R signaling. In the presence of NHERF1 and NHERF2 the activated PTH1R may switch from AC signaling to PLC signaling (46, 48).

Evidence also exists to suggest that there may be other receptors for the amino-terminal fragment of PTH. These receptors however, are believed to be expressed in

non-classical PTH target tissues (i.e. non-kidney, non-skeletal) and have yet to be identified (49).

PTH2 Receptor (PTH2R)

Unlike the PTH1R, the PTH2R is expressed in relatively few tissues throughout the body. Some tissues shown to express the PTH2R include the placenta (50), blood vessels (51) , and central nervous system (50, 52). This receptor was cloned due to its structural similarity to the PTH1R, and although the human PTH2R is activated by amino-terminal PTH, this is not the case in other species that have been studied. In fact, the PTH2R is likely not a "true" PTH receptor at all. This is because it is believed that PTH is not its natural endogenous ligand. Instead, it is thought that a small peptide know as the hypothalamic tuberoinfundibular peptide of 39 residues (TIP 39) is the primary natural ligand for the PTH2R, not PTH (4, 43). Many of the biological functions mediated through the PTH2R are still unknown, but reports to date have shown no role for it in mineral homeostasis; instead, it may be important in the regulation of renal blood flow (4, 51), and to pain responses (43, 53). Although the PTH2R may not be relevant for the biological actions of PTH, it was included here for completeness.

Carboxy-terminal PTH Receptor (C-PTHR)

There is evidence that a receptor exists which binds carboxy-terminal (C-terminal) PTH fragments with high affinity. This receptor has yet to be cloned, and its biological role is not well understood and still requires much investigation (43, 46, 54).

However, studies have suggested that C-terminal PTH fragments, which do not bind to the PTH1R to initiate the classical effects of PTH, actually bind to the C-PTHR to exert biological actions that are opposite to that of PTH [1-34] binding to the PTH1R (43, 55, 56). For example, unlike PTH [1-34], C-terminal PTH fragments have been shown to inhibit bone resorption (57) and to decrease blood calcium concentrations (58). However, since a C-terminal knockout animal has yet to be made, it remains unknown how relevant these actions are to calcium homeostasis overall.

E. Parathyroid Hormone Related Protein (PTHrP)

Human PTHrP is encoded by a gene located on Chromosome 12 (59), while the mouse PTHrP gene is located on Chromosome 6 (60). PTHrP is widely expressed in both developing and adult tissues, including: cartilage, bone, mammary glands, the central nervous system and the placenta (61, 62). However, unlike the endocrine hormone PTH, in the adult PTHrP is normally undetectable in the circulation because it functions principally in a paracrine/autocrine manner in many cells and tissues (49). Compared to PTH, an 84 amino acid peptide, PTHrP is much longer. In fact, in humans it has three isoforms: 139, 141, and 173 amino acids in length, which result from alternative RNA splicing. In these isoforms, amino acids 1-139 are identical (49, 61, 62). In the mouse however, PTHrP is a single 139 amino acid chain (49).

Cells which secrete PTHrP cleave full-length PTHrP to produce at least 3 known forms of secreted peptides. These include the amino-terminal fragment, or PTHrP [1-36]; the mid-region fragment, or PTHrP [38-94]; and the C-terminal fragment, or PTHrP

[107-139] (49, 61, 62). The biological functions of each of these fragments are unique and believed to function through distinct receptors.

1. PTHrP Receptors and Signaling

PTH1R

Like PTH [1-34], PTHrP [1-36] also binds to the PTH1R (49, 61, 62). Both PTH [1-34] and PTHrP [1-36] mediate classical effects on calcium homeostasis when they bind and activate the PTH1R (49). As a result, it is a complex task to delineate the relative roles of PTH and PTHrP in calcium and bone metabolism. In order to explore the role of PTH in fetal-placental calcium and bone metabolism, PTHrP must also be taken into consideration.

It has generally been believed that the amino-terminal fragments of PTH and PTHrP bind to the PTH1R with equal affinity and exert effects on mineral ion homeostasis with equal potency via identical mechanisms (49). More recently however it has become clear that PTH [1-34] and PTHrP [1-36] can differ in how they bind to the PTH1R. It appears that the PTH1R has the capacity to form various conformational states and bind the two ligands with different affinities. In one conformational state it had been shown that PTH binds with greater affinity and can mediate prolonged signaling (for hours), while PTHrP binds for only minutes. Thus, in this instance, PTH can cause prolonged signaling responses in bone and/or kidney PTH target cells, which can ultimately lead to different biological outcomes (63, 64).

As with PTH, previous studies have suggested that other receptors exist which bind the amino-terminal fragment of PTHrP. In zebrafish a receptor (PTH3R) has been identified that selectively binds PTHrP [1-36] (65). Whether a homolog of this receptor exists in mammals remains unknown.

Thus to review, both amino-terminal fragments of PTH and PTHrP activate the PTH1R, while the PTH2R binds PTH but not PTHrP, and the PTH3R binds PTHrP but not PTH.

Receptors for the mid-region and C-terminal fragments of PTHrP

It has been reported that C-terminal regions of PTHrP, including PTHrP [107-139] which has been named osteostatin (62), in contrast to the actions of PTHrP [1-36], can inhibit osteoclastic bone resorption (66), while the mid-region of PTHrP has been shown to stimulate placental calcium transfer (67-70). It is known that both of these fragments do not bind to the PTH1R and have no PTH-like functions. However, even though there is strong evidence to suggest that both mid and C-terminal fragments of PTHrP have distinct receptors, they have yet to be identified (49).

F. Mouse Models of PTH and/or PTHrP Deficiency

Several knockout mouse models of PTH and/or PTHrP deficiency have been created, and they serve as useful models to explore the role of PTH in fetal-placental calcium and bone metabolism. Each of the models vary with respect to how the function of PTH and/or PTHrP is disrupted in the mouse, and as a result they offer insight into the

role of not only PTH, but also PTHrP and the parathyroids, in regulating fetal-placental calcium and bone homeostasis.

Below, several of the relevant knockout mouse models that have previously been explored are listed (see **Table 1** for a summary of previously published data from the various knockout mouse models). A model that had yet to be examined with regards to fetal-placental calcium and bone metabolism, the *Gcm2* null, is also introduced because it will be utilized in the present study. Subsequent sections will discuss what has already been learned from these models, particularly with respect to the role of PTH in regulating fetal blood calcium, skeletal development and mineralization, and placental calcium transfer, and what questions still remain.

1. *Homeobox A3* (*Hoxa3*) Null

Deletion of the *Hoxa3* gene results in a range of abnormalities in the pharyngeal tissues, particularly those arising from the 3rd and 4th pharyngeal arches (71). As a result, *Hoxa3* null mice are lacking parathyroids (aparathyroid) and circulating PTH (71, 72). Furthermore, they lack the thymus (athymic) and have thyroid hypoplasia. Deletion of the *Hoxa3* gene is lethal at birth. (71).

2. *Parathyroid Hormone* (*Pth*) Null

Pth null mice have a normal thymus and thyroid. The parathyroid glands are present but enlarged: they completely lack PTH because the gene encoding PTH has been

deleted. Deletion of the *Pth* gene is not lethal prior to birth (73), but many presumably hypocalcemic deaths occur in subsequent weeks (Unpublished).

Although the fetal bone phenotype has previously been evaluated in the *Pth* nulls in a C57BL/6 inbred strain background, placental calcium transfer and other parameters of calcium metabolism (e.g. blood ionized calcium and plasma PTHrP levels) had not yet been assessed.

3. *Parathyroid Hormone-related Protein* (*Pthrp*) Null

Pthrp null mice have normal thymus, thyroid, and parathyroids. They completely lack PTHrP because the gene encoding PTHrP has been deleted. Most *Pthrp* null mice die within the first 30 minutes after birth, but occasionally pups survive several days. The cause of death may be from a variety of causes including hypocalcemia, restrictive lung disease (rigid rib cage), and acute respiratory distress syndrome due to surfactant deficiency (74-76).

4. *Type 1 PTH/PTHrP Receptor* (*Pthr1*) Null

Pthr1 null mice have high circulating levels of both PTH and PTHrP. However, because the PTH1R, which binds the amino-terminal region of both PTH and PTHrP, is deleted, the biological effects of the amino-terminal of PTH and PTHrP are absent while mid and C-terminal effects are retained. Depending on the genetic background that the *Pthr1* null mice have been backcrossed into, they die between mid-gestation and immediately after birth (67, 75).

5. *Glial Cells Missing 2* (*Gcm2*) Null

Gcm2 encodes a transcription factor whose expression is essential for the development of the parathyroid glands. Thus, *Gcm2* null mice lack the parathyroid glands but have a normal thymus and thyroid. Despite lacking parathyroid glands and being hypocalcemic and hyperphosphatemic, *Gcm2* null mice were reported to have normal circulating levels of PTH. It was speculated by Gunther *et al.* (2000) that the auxiliary source of PTH in the *Gcm2* nulls was coming from a small cluster of PTH-producing cells in the thymus. However, they did not attempt to find PTH-producing cells in the thymus of the *Gcm2* null mice. Rather, they showed that there was a small cluster of PTH-producing cells in the thymus of WT mice that had previously been thyroparathyroidectomized. In turn, they assumed that such PTH-producing cells must be present in *Gcm2* null thymus.

It is known that the parathyroids develop with the thymus from a bilateral common primordium in the third pharyngeal pouch in mouse embryos at about day 11 of gestation. By about day 13.5 of gestation this primordium separates into one parathyroid gland lobe and one thymus lobe on each side (26, 77, 78). This primordium intensively expresses *Gcm2* (78). Several years after the *Gcm2* nulls were studied by Gunther *et al.* and the start of this project, Liu *et al.* (2007) reported that neither PTH mRNA or PTH protein expression were present in the common parathyroid/thymus primordium in the *Gcm2* null mice on embryonic day 11.5-12.5, while both were clearly present in the WT mice. Moreover, unpublished work from these authors has demonstrated that parathyroid tissue which is present in the thymus of WT mice appears to be the result of fragments of

parathyroids that become detached during the developmental migration of the embryonic parathyroids as opposed to being PTH-producing cells that have derived *de novo* from the thymus. Thus, this work predicts that *Gcm2* nulls should not have parathyroid cells in the thymus at all. Overall, based on this subsequent research and the fact that Gunther *et al.* (2000) failed to directly show that a small group of cells in the thymus of the *Gcm2* nulls was responsible for producing PTH, it remains puzzling why the *Gcm2* nulls had "normal" circulating PTH levels and where it was coming from.

From *Gcm2* HET male and female matings it was reported that 30% of the *Gcm2* null offspring die shortly after birth. The remaining nulls were viable and fertile (79).

Unlike the previously discussed knockouts, fetal-placental calcium and bone metabolism in the *Gcm2* null fetal mice had not yet been explored before I began this project.

G. Regulation of Blood Calcium in the Fetus

As noted previously, it is well established that fetal blood calcium is maintained at a level higher than in the mother. However, it is unknown exactly how early in gestation this occurs. In humans, fetal hypocalcemia has been reported as early as 15-24 weeks of gestation (80), while in mice it has been observed as early as day 15 of gestation which is the earliest time point that a sufficient blood sample can be collected for the assay (unpublished data, Kovacs lab). Remarkably, the ability of the fetus to maintain a blood calcium level higher than the mother is robust because even in the face of chronic, severe maternal hypocalcemia, fetal blood calcium remains unaffected (31). For example, *Vdr*

null fetal mice had normal ionized calcium levels, despite having *Vdr* null mothers that were severely hypocalcemic (33). The biological significance or role of the high fetal blood calcium is unclear. It has been suggested that it may protect against severe neonatal hypocalcemia (20). Under normal circumstances, in the first 12 hours after birth, the neonatal blood calcium level drops significantly below the maternal level before it eventually rises to the normal adult concentration (31). Thus, by starting at a higher than maternal level, the fetal blood calcium may serve to protect the fetus from dropping to a severely low level after birth (20). Historically, the high fetal blood calcium level has also been suggested to be necessary for the fetal skeleton to mineralize. Prior studies have suggested that to mineralize the fetal skeleton a blood calcium equal, but not above, the maternal level is sufficient (20, 29, 31, 34, 81).

As previously discussed, in the adult it is the CaSR which directly regulates the blood calcium level by controlling the secretion of PTH into the circulation. Similarly, in the fetus it also appears that the CaSR regulates the secretion of PTH. This is because deletion of the CaSR leads to a stepwise increase in PTH, which suggests that the CaSR probably suppresses PTH in response to high fetal blood calcium. Deletion of the CaSR however also leads to an increase in ionized calcium. Thus indicating that the CaSR is not responsible for setting the high blood calcium level that is normally observed in the fetuses because in its absence, the blood calcium level becomes even high (15).

The high fetal blood calcium level appears to be directly regulated by both PTH and PTHrP. This is based on the observations that in the absence of PTHrP (*Pthrp* null fetal mice), fetal blood calcium was reduced from its normal high level to the maternal level

(67), while in the absence of PTH and the parathyroids (*Hoxa3* null fetal mice) the fetal blood calcium dropped significantly below the maternal level (72). Furthermore, in the absence of PTH, the parathyroids and PTHrP (*Hoxa3/Pthrp* null fetal mice) the blood calcium level declined to a level that was even lower than that observed in either of the single mutants (29), and equal to that observed in mice lacking the PTH1R (29, 67) (**Figure 3**). Thus, both PTH (and the parathyroids) and PTHrP independently contribute to blood calcium in the fetus and one hormone cannot compensate for the lack of the other; in the absence of either the blood calcium is reduced (20, 81, 82). Although PTH circulates at much lower levels in the fetus as compared to PTHrP, it appears to play a larger role in regulating the blood calcium because absence of PTH (and parathyroids) caused a greater fall in blood calcium than PTHrP alone.

In response to hypocalcemia and lack of PTHrP, PTH increases. In *Pthrp* null fetuses, serum PTH levels were reported to be increased approximately 3-fold as compared to that of WT fetuses. This increase in PTH may have prevented more severe hypocalcemia (29), but PTH may have been be unable to compensate completely for absence of PTHrP because it is restrained by the actions of the parathyroid CaSR's which allow PTH to increase the blood calcium to the normal adult level, but not higher (i.e. to the normal fetal level). Unlike PTH however, PTHrP may not respond in the same manner to lack of PTH and fetal hypocalcemia. *Hoxa3* null fetuses (completely lacking PTH), despite having more profound hypocalcemia (below the maternal level), do not have an increase in plasma PTHrP (72).

It is uncertain how PTH and PTHrP independently and concurrently regulate blood calcium through the single PTH1R. It has been suggested that both ligands may actually not function through the PTH1R to regulate fetal blood calcium. Instead, PTH may regulate blood calcium by acting on classic target tissues such as kidney and bone which express the PTH1R, while it may be the mid-region of PTHrP that contributes to blood calcium through binding to a distinct receptor that regulates placental calcium transfer (20, 81, 82).

The preceding discussion has relied heavily on data from *Hoxa3* nulls which in addition to lacking PTH and the parathyroid glands have other problems that may confound their phenotype. They have abnormalities in tissues deriving from the third and fourth pharyngeal arches which result in the absence of the thymus (83). Thus, it is unclear whether the phenotype observed in the *Hoxa3* null fetuses is solely the result of absence of PTH, whether absence of the parathyroid glands is a contributing factor, or even if other abnormalities in the *Hoxa3* nulls contribute to the calcium and bone phenotype.

See **Table 1** for a summary of previously published data from the various knockout mouse models showing the effect of PTH and/or PTHrP deficiency on fetal blood calcium.

H. Regulation of Skeletal Development and Mineralization in the Fetus

The fetal skeleton must undergo ample growth and significant mineralization prior to birth (20, 81, 82). Previous studies in fetal sheep have suggested that the fetal

parathyroids are necessary for these processes to occur normally because fetal parathyroidectomy and thyroparathyroidectomy resulted in fetal skeletons that were rachitic and lacking calcium (84, 85). Studies in fetal rats however, showed that the fetal parathyroids are not required because fetal decapitation (thought to mimic thyroparathyroidectomy) did not adversely affect the fetal skeleton (86).

In the time since the surgical models above were carried out, comparative studies on various genetic knockout mouse models have been utilized to sort out the relative roles of the parathyroids, PTH and PTHrP in fetal skeletal development and mineralization. Studies on *Pthrp* nulls, which lack PTHrP, revealed that although the fetal skeletons were fully mineralized (29), they suffered from numerous abnormalities of endochondral bone formation due to premature terminal differentiation of chondrocytes in the growth plate (74). The absence of PTH (and parathyroids) however, in the *Hoxa3* null fetal mice, resulted in normal long bone and growth plate development, but the skeleton was significantly undermineralized (29). Removing PTH (and parathyroids) and PTHrP, as in the *Hoxa3/Pthrp* null, resulted in smaller fetuses with a more severe *Pthrp* null chondrodysplasia and undermineralized skeletons (29). Essentially, the phenotype of *Hoxa3* nulls was superimposed on that of *Pthrp* nulls. Thus, it appears that for normal development of the cartilaginous skeletal scaffold which is sequentially resorbed and replaced with the bone matrix, PTHrP is required, while PTH or functioning fetal paratyhroids are required for the fetal skeleton to become mineralized before birth (20). More specifically, it is thought that PTHrP acts locally in the growth plate to direct the development of the cartilaginous skeletal scaffold (87), whereas PTH mineralizes the

fetal skeleton by maintaining sufficient circulating levels of minerals, such as calcium (29) and by direct actions on osteoblasts to stimulate bone formation. Furthermore, it is believed that in order for the fetal skeleton to mineralize sufficiently, the fetal blood calcium concentration must be maintained at a level that is at least equal, but not necessarily higher, to the maternal blood calcium level (20).

Subsequent to the *Hoxa3* null studies discussed above, studies involving *Pth* null mice were reported. Like the *Hoxa3* null mice, *Pth* null mice also had undermineralized skeletons (73). However, unlike the *Hoxa3* nulls which were reported to have no skeletal abnormalities beyond skeletal undermineralization, the *Pth* nulls showed quite a different phenotype with slightly shortened long bones, abnormally formed skulls, smaller vertebral bodies, and shorter metacarpal and metatarsal bones. Furthermore, with closer examination it was also found that among many other abnormalities, the long bones of the *Pth* nulls also had enlarged hypertrophic zones, increased cortical thickness, and decreased osteoblast number (73). With the phenotypes varying so significantly between the two models of absence of fetal PTH, the role of PTH in fetal skeletal development and mineralization became less certain. The phenotypic differences observed in the above studies may have resulted because both mouse models were studied in different genetic backgrounds. *Hoxa3* null mice were studied in the background of Black Swiss (29), while the *Pth* null mice were studied in a C57BL/6 background (73), which are known to have a lower blood calcium level as compared to Black Swiss mice (67). The phenotypic differences between the two knockout mouse models are quite interesting. The *Pth* nulls lacked only one thing (i.e. PTH) but had abnormal skeletons, while the *Hoxa3* nulls

lacked multiple things (i.e. PTH, parathyroids, thymus) but had normal skeletons (albeit undermineralized). This leads to the question: how could multiple abnormalities add up to a normal skeleton, and only one abnormality cause an abnormal skeleton? This very puzzle made it necessary to examine the two models in the same genetic background.

See **Table 1** for a summary of previously published data from the various knockout mouse models showing the effect of PTH and/or PTHrP deficiency on fetal skeletal development and mineralization.

I. Placental Calcium Transport

1. The Placenta

The placenta assumes many functions that are carried out by separate organs in the adult body. It acts as the fetal lungs, disposes of fetal waste, is a site of hormone production, and performs many other functions. Perhaps the most fascinating aspect of the placenta however, is its architecture. It is comprised of cells from two genetically distinct organisms; the mother and the fetus (40).

The structure of the placenta varies considerably among different species. They have various shapes, cellular organizations and different patterns with respect to how it is distributed over the uterine lining (40, 88) . Both sheep and rodent placenta have contributed to our knowledge of the placental unit and the factors that regulate placental calcium transfer. Thus, it is important to be aware of the various types of placenta, how they differ, and how they compare to the human placenta because all these factors may

affect our understanding of how the placenta functions, and how, or if, findings from animal studies can apply to humans.

The placenta of sheep, based on gross morphology, can be classified as a cotyledonary placenta. This means that the placental tissue is restricted to numerous (60-70 in a single pregnancy) individual elements know as cotyledons, which are spread over the entire uterine wall. Rodents and humans however have the most localized type of placenta, known as discoid, in which the placental tissue is confined to a single plate that attaches to the uterine wall (40, 88).

Placentas can also be classified according to the number of cell layers that separate the maternal and fetal blood, which also reflects the degree to which the trophoblast layer of the embryo invades the maternal uterine tissue. Based on these elements, sheep placenta is considered to be epitheliochorial. Six layers of tissue separate the maternal and fetal blood because the uterine cell layer remains intact; it is not invaded by the fetal trophoblasts at all **(Figure 4A)**. In contrast, humans and rodents have a haemochorial placenta. This type of placenta shows the greatest extent of fetal trophoblast invasion into the maternal uterine tissue, which results in the maternal blood being in direct contact with the fetal chorion layer **(Figure 4B)** (40, 88).

Mice are great experimental models for studying placental development and function as the overall structure of human and rodent placenta (both discoid and haemochorial) are very similar. However, there are some structural differences between the rodent and human placenta that should be noted. First, although both have a haemochorial type placenta, rodents are further classified as being haemotrichorial, meaning it has three

layers of placental trophoblast cells (labyrinthine trophoblasts (i.e. Syncytiotrophoblasts), spongiotrophoblasts, and giant trophoblasts) that separate the maternal blood from the fetal blood. The human placenta however is haemomonochorial, as it has a single trophoblast cell layer separating the maternal and fetal blood (88-90). A second difference between the human and mouse placenta exists in the area of the placenta in which the fetal and maternal blood comes in closest contact with one another. In humans, tree-like structures, know as villi, protrude from the fetal side into the intervillous space, which is filled with maternal blood, allowing the villi to "float" in the large blood-filled space. In rodents however, instead of the large blood-filled space, there is a labyrinth. Thus, instead of the villi floating freely in the maternal blood, the villi are surrounded by a series of small channels that are filled with maternal blood (91). Another difference between the human and rodent placenta is that the rodent, but not the human placenta, contains a unique structure known as the intraplacental yolk sac (IPYS). The IPYS is a bilayered membrane that is located between the fetal blood vessels and the maternal blood filled channels. Overlying the fetal vessels are tall columnar cells, while on the opposite side, small parietal (or cuboidal) cells overlie the maternal blood spaces and a thick basement membrane know as Reichert's membrane **(Figure 5)**. This structure has been shown to highly express many calcitropic hormones, receptors, and channels, including calcium adenosine triphosphatase (Ca^{2+}-ATPase), CaSR, PTH1R, VDR, calbindin-D9k (CaBP9k), and transient receptor potential vanilloid 6 (TRPV6). Furthermore, a decrease in calcitropic hormones in this structure, for example the decrease in CaBP9k in the *Pthrp* null fetuses, coincides with a decrease in placental

calcium transfer (92). Therefore, due to its well situated positioning and calcitropic gene expression it is likely that the IPYS is a site of maternal-fetal calcium exchange (20, 92, 93).

2. Techniques for Assessing Placental Calcium Transport

Various techniques have been utilized to study the process of placental calcium transport. The techniques are quite different from one another, and each offers its own benefit and limitations. The placental perfusion technique has been utilized in sheep (94, 95), rat (96, 97), and more recently, mouse (98, 99) studies. This technique involves removing the fetus from the uterus and attaching the placenta via the umbilical vessels to a semi-closed circuit. The placenta is then perfused *in situ* with either actual fetal blood or a blood substitute, while the flow rate and perfusion pressure of the system is kept constant (**Figure 6**). Depending on what is being studied, the system can then be altered accordingly. For example, to test the effect of a specific peptide on placental calcium transfer, the peptide of interest can be injected directly into the portion of the circuit that is connected to the umbilical arteries that will flow into the placenta (in-flow), while continuous sampling to measure changes in the calcium concentration can be taken from the portion of the circuit that is coming from the placenta via the umbilical vein (94). Similarly, to actually measure the rate of calcium transfer, both ^{45}Calcium (Ca) and ^{51}Chromium (Cr)-Ethylenediaminetetraacetic Acid (EDTA) can be administered into the in-flow of the circuit, and the clearance of ^{45}Ca relative to ^{51}Cr-EDTA can be monitored to determine the rate of calcium transfer (96). This method has also been utilized to

indirectly (because the fetus is removed) study the contribution of the fetal parathyroids to placental calcium transfer. In fetal lamb studies, this was accomplished by performing a fetal thyroparathyroidectomy, then supplementing the fetus with thyroid hormone for a period of time before the placental perfusion circuit was set up to assess placental calcium transfer (94, 95). In rat studies, to simulate a thyroparathyroidectomy, decapitation was performed, and a couple of days later the fetuses were removed and the placenta were perfused *in situ* to evaluate the effect of lack of parathyroids on placental calcium transport (96). In recent years, the placental perfusion technique, with some adaptations, has been utilized in mice. Due to the small nature of mice, in order to successfully place a catheter in the umbilical vessel it is necessary to dilate the vasculature with nitroglycerin. Furthermore, due to the small volume of blood in the mouse, unlike studies in rats and sheep, numerous maternal blood samples cannot be collected over the course of the experiment. Thus, the maternal radioisotope concentration, which is utilized in a formula to determine the rate of transfer across the perfused placenta, is determined from a maternal radioisotope disappearance curve that was created from collecting single samples from different dams at varying time points, rather than collecting serial maternal blood samples at various time points throughout a single experiment (98, 99).

Another very different technique, which allows the fetus to remain intact, has also been utilized in mice to assess placental calcium transfer. This technique involves giving the pregnant mother an intracardiac injection containing ^{45}Ca and ^{51}Cr-EDTA. 5 minutes after the maternal injection, the fetuses are removed, and the amount of each isotope that

has accumulated within each fetus is determined. Because ^{51}Cr-EDTA only crosses the placenta by passive diffusion it serves as a control for differences in flow rate between the individual placentas in one litter, ^{45}Ca however is actively transported across the placenta from mother to fetus as well as crossing passively. Thus, by correcting for the rate of diffusion (using the ^{51}Cr-EDTA) the relative rate of placental calcium transfer in each fetus in the litter can then be determined (67). Unlike fetal surgery or decapitation that was used in combination with the placental perfusion technique (discussed above) to assess the contribution of the fetal parathyroids on placental calcium transfer, this technique utilizes knockout mice. For example, in previous studies male and female mice HET for the *Hoxa3* gene were mated such that litters would contain WT, HET and *Hoxa3* null fetuses, which lack the parathyroids. Pregnant mothers were then given the intracardiac injection of ^{45}Ca and ^{51}Cr-EDTA, and placental calcium transfer was then assessed to see if it differed between genotypes (72). Furthermore, using this technique to assess the effect of specific hormones on the rate of placental calcium transfer is quite different than using the placental perfusion technique. The testing of various hormones can be accomplished through performing a laparotomy and administering an intraabdominal injection of the test peptide to the fetus *in utero*, prior to giving the mother the radioisotope intracardiac injection to assess the rate of placental calcium transfer (67).

In summary, the techniques that have been used to assess placental calcium transfer differ significantly. Therefore, when reviewing placental calcium transfer studies it is very important to differentiate between the techniques that have been used, and keep in

maintain the normal fetal blood calcium level but also having to mineralize the fetal skeleton. This is a great demand placed on the fetus because the mineralization occurs very rapidly, with 80% of the required mineral being accreted in the third trimester in humans (7) and 95% of the required mineral being accreted in the final 5 day of gestation in rats (8). In humans, it is likely that the site of active transport is the fetal-facing basement membrane of the syncytiotrophoblast cells, while in rodents it is likely the labyrinthine trophoblasts and the basal surface of the endoderm of the IPYS (20, 31, 92). At these sites, it is thought that (similar to calcium transfer across the intestinal mucosa and kidney) (100), calcium enters through calcium channels, such as TRPV6, at the maternal-facing basement membrane, is then carried across the cell by calcium binding proteins, such as calbindin-D9k, and finally actively expelled at the fetal-facing basement membrane by Ca^{2+}-ATPase (i.e. the calcium pump) (20, 81, 82, 92, 101) **(Figure 7)**.

Although it is clear that active placental calcium transfer is an important process in fetal calcium homeostasis, the components and regulating factors have not been fully elucidated. It is thought that both the maternal and fetal factors contribute to regulating this process (20).

Maternal Regulation

Maternal hormones may influence the process of fetal-placental calcium transport by either acting directly on the placenta or by altering (increasing or decreasing) the maternal blood calcium level. However, studies in animal models have suggested that despite maternal hypocalcemia or hormone deficiencies, the normal maternal-to-fetal

calcium transfer can usually be sustained (20, 31, 81, 82). For example, placental perfusion studies in sheep found that the rate of placental calcium transfer was unaffected by maternal hypocalcemia caused by either a parathyroidectomy or by a restricted calcium diet (94, 102). Likewise, in fetal mice, it was discovered that ablation of *Vdr* did not adversely affect placental calcium transfer, even though the *Vdr* null mothers suffered from severe hypocalcemia (33). The fact that the rate of placental calcium transfer is not negatively impacted under these circumstances does not necessarily mean that the fetus is unaffected by maternal hypocalcemia. It is likely that because of the low maternal calcium concentration, the fetal-placental unit may be working much harder to pump the normal amount of calcium across the placenta to the fetus. Furthermore, although not evident in rodents, in humans, maternal hypocalcemia due to hypoparathyroidism has been associated with problems such as fetal parathyroid hyperplasia, skeletal demineralization, intrauterine fractures, and bowing of the long bones, which are all symptoms consistent with intrauterine fetal hyperparathyroidism (103, 104).

Fetal Regulation

Several animal models have been utilized to explore the potential role of fetal hormones, and their receptors, in regulating placental calcium transfer. The receptor for 1, $25(OH)_2D_3$, VDR, has been shown to be expressed in the placenta of sheep (105), humans (106, 107), rats (108), and mice (92). Furthermore in mice, the VDR has been shown to be expressed highly in the IPYS as compared with trophoblasts (92). The VDR is also known to be involved in calcium transport in both the intestine (109, 110) and

kidney (110). Based on the above findings, it is reasonable to infer that the VDR is involved in calcium transport across the placenta. This idea has been supported by several studies. First, it was reported that in sheep, 1,25-$(OH)_2D_3$ partly restored reduced calcium transfer (as assessed by the placental perfusion technique) that resulted from a nephrectomy (101). Furthermore, in other placental perfusion studies in rats (111) and sheep (112), it was reported that pharmacological doses of 1,25-$(OH)_2D_3$ or 1α-hydroxycholecalciferol increased calcium transfer. Contrary to these findings however, studies in mice have concluded that it is unlikely that the VDR or 1,25-$(OH)_2D_3$ (which was indirectly tested) plays a role in regulating placental calcium transfer. This conclusion was based on the findings that *Vdr* null fetal mice had a normal rate of placental calcium transfer, normal levels of many calcitropic hormones, and normal placental expression of CaBP9k, which is thought to play an important role in maternal-fetal calcium transfer in late gestation (33, 92, 101). Thus, it was theorized that $1,25(OH)_2D_3$ is likely not required for the normal regulation of placental calcium transfer, at least in mice (20, 33), since the ablation of fetal VDR did not alter placental calcium transfer. However, in the above *Vdr* null studies, placental calcium transfer was non-significantly increased in the *Vdr* null fetal mice. (33). This may indicate that the role of VDR is to actually decrease, rather than increase, placental calcium transfer.

The role of the parathyroid glands in regulating placental calcium transfer has also been examined. In thyroparathyroidectomized fetal sheep (supplemented with thyroid hormone) (94, 95), and in decapitated fetal rats (to approximate a thyroparathyroidectomy) (96), placental calcium transfer was found to be reduced as

assessed by the placental perfusion technique (94-96). Together, these results lead to the conclusion that the parathyroids play an important role in active maternal-fetal calcium exchange. Furthermore, in the fetal thyroparathyroidectomized sheep studies, when autologous blood from intact fetal sheep (i.e. which have parathyroid glands) was infused into the perfusion circuit, calcium transfer across the perfused placenta of the thyroparathyroidectomized fetal sheep was restored (95). In similar sheep studies however, it was discovered that PTH did not restore calcium transport (70). Therefore, it appeared likely that the parathyroids might be producing something other than PTH that regulated placental calcium transfer (70). In fact, it was thought that it may be PTHrP that was responsible for regulating placental calcium transfer. PTHrP [1-141], PTHrP [1-86] and PTHrP [67-86] all stimulated placental calcium transfer whereas PTH was without effect. The collective results seemed to indicate that it was the mid-molecular form of PTHrP, PTHrP [67-86], that was somehow active in regulating the transfer of calcium across the placenta, because PTHrP [1-34], like PTH, had no effect (68-70). The overall results from studies on thyroparathyroidectomized fetal sheep led to the theory that PTHrP, which may be produced by the parathyroid glands, is a crucial factor in regulating placental calcium transfer, while PTH is not. Although it was concluded that it was PTHrP from the parathyroids that stimulated calcium transfer, this is not certain. Circulating levels of PTHrP were not measured in these studies, and thus it remains unknown whether the levels of PTHrP were reduced as a result of the thyroparathyroidectomy.

Studies in fetal mice further examined the role of PTHrP in the process of placental calcium transfer. Similar to the studies on sheep, these studies also implicated PTHrP, more specifically the mid-molecular region of PTHrP, to be responsible for stimulating placental calcium transfer. Furthermore, as assessed not by the placental perfusion technique, but rather by examining intact fetal mice, it was found that *Pthrp* null fetal mice had reduced placental transfer of calcium, but when treated with PTHrP [1-86] or PTHrP [67-86] calcium transfer increased. Treatment with PTHrP [1-34] or PTH [1-86] however, had no effect (67).

One confounding piece of evidence against the role of PTHrP in regulating placental calcium transfer comes from a study on *Pthrp* null mice. Contrary to the study discussed above, it was reported that placentas from *Pthrp* null fetuses actually showed an increase in the rate of calcium transfer across the placenta as compared to placentas from both WT and *Pthrp* +/- fetuses (98). In this study, calcium transfer was assessed by the placental perfusion technique adapted for mice. It is likely that this accounts for the opposite results observed between this study and that which assessed placental calcium transfer in intact *Pthrp* null fetuses. This is due to the fact that PTHrP is expressed in the smooth muscle vasculature and exerts effects on vascular development and physiology (113). Thus, it is likely that the vasculature of the *Pthrp* null mice is different from that of WT mice. As a result, dilating the vasculature with nitroglycerin prior to measuring placental calcium transfer may have resulted in different responses between genotypes (e.g. the *Pthrp* nulls may dilate more than WT or HET fetuses), and affected the rate of placental calcium transfer observed in each. Furthermore, not only are the fetuses absent

in this method, but there is only one placenta examined per pregnancy. Thus, there are no *in utero* littermates for the null to be compared to, which would allow the affect of the vasodilator to be taken into account. Instead, the method assumes that the blood flow from mother to fetus is the same from mother to mother. This however, may not be the case.

As with fetal sheep studies, studies on *Pthrp* nulls did not help determine if the fetal parathyroids produce PTHrP, or if parathyroid derived PTHrP could be the source that is important for placental calcium transfer. This is because *Pthrp* null fetuses do not produce PTHrP in any tissues. Studies in other genetically altered mice however, may be informative. As previously mentioned, *Hoxa3* null fetuses completely lack PTH, and are aparathyroid and athymic (83). In spite of this, they had a normal rate of placental calcium transfer and unaltered plasma PTHrP levels (72). In addition, *Pthr1* null fetuses (which lack the PTH1R) had an increased rate of placental calcium transfer and elevated plasma PTHrP levels. It was found however, that PTHrP mRNA was upregulated in both the placenta and liver, but not the neck region that would contain the parathyroids (72). Thus, in contrast to fetal sheep studies, studies in fetal mice suggested that the fetal parathyroids do not produce PTHrP. This is consistent with a rat study which reported that PTHrP was not detectable in the fetal parathyroids, as assessed by *in situ* hybridization, immunohistochemistry, and RT-PCR (114).

Despite the evidence in favor for a role of PTHrP, but not PTH, in stimulating placental calcium transfer, there is also evidence suggesting that PTH may indeed stimulate placental calcium transfer. In rat decapitation studies, as discussed above, it was

found that PTH did stimulate placental calcium transfer (96). Furthermore, it has been reported that vesicles created from human syncytiotrophoblast basal membranes *in vitro,* increased calcium accumulation when treated with PTH [1-34] (115). The fact that the PTH1R is highly expressed in the placenta, particularly in the IPYS which is believed to be a site of maternal-fetal calcium exchange (67, 92), also suggests that PTH may play a role in regulating placental calcium transfer; especially since the PTH1R is not the receptor that the mid-region of PTHrP acts on to stimulate placental calcium transfer (67). Furthermore, the previously discussed studies carried out by Kovacs *et al* (1996) showed that treating *Pthrp* null fetuses *in utero* with PTHrP [67-86] increased calcium transfer while treatment with PTH [1-86] did not. It was later discovered that *Pthrp* null fetuses had a significant increase in endogenous circulating PTH (29). Thus, treating the fetuses with exogenous PTH may have been ineffective if the fetuses were already maximally upregulating placental calcium transfer in response to the high endogenous PTH.

As can be seen from the research reviewed above, there are discrepancies among various animal models and studies regarding the role of the parathyroids in placental calcium transfer, the source of PTHrP that is important for stimulating placental calcium transfer, and the role, if any, of PTH in regulating placental calcium transfer. These differences may be true species differences, or they may be due to methodological differences. The experimental design in the current study, which is discussed in detail in the next section (i.e. **II. Project Description, Purpose and Hypothesis**), allows some of

these issues to be addressed and will help clarify some of the inconsistencies that currently exist in the literature.

See **Table 1** for a summary of previously published data from the various knockout mouse models showing the effect of PTH and/or PTHrP deficiency on placental calcium transfer.

II. Project Description, Purpose and Hypothesis

As can be seen from the previously reviewed literature, studies on genetically engineered mouse models have provided great insight into the role of PTH in fetal-placental calcium and bone homeostasis. Despite the efforts however, the role of PTH remained unclear. Therefore, the purpose of the present doctoral research was to further examine the role of PTH in fetal-placental calcium and bone homeostasis and test the hypothesis that: *PTH, despite its low circulating levels during fetal life, plays an important role in regulating not only fetal blood calcium and skeletal development, but also placental calcium transfer.* To test this hypothesis, several remaining questions regarding the role of PTH in fetal calcium homeostasis were addressed:

1) Is the phenotype of the *Pth* null fetal mice in the Black Swiss background the same as the *Pth* null fetal mice in the C57BL/6 background?
2) Does absence of PTH have the same consequences on skeletal mineralization and blood calcium as absence of PTH and parathyroids? If not, why does the phenotype of the *Pth* nulls differ from that of the *Hoxa3* nulls?
3) Does PTHrP increase in response to fetal hypocalcemia and/or absence of PTH?
4) Does PTH regulate placental calcium transfer?
5) Does PTH regulate placental gene expression?
6) Is PTH expressed locally in the placenta?

To help answer these questions, two mouse models were utilized: the *Pth* knockout and the *Gcm2* knockout. These models served as excellent tools for addressing

the above questions. The *Gcm2* nulls served as an intermediate model between the *Pth* nulls and the *Hoxa3* nulls: *Hoxa3* nulls, have absence of PTH and parathyroids; *Pth* nulls, have absence of PTH but retention of parathyroids; *Gcm2* nulls, have absence of parathyroids, but retention of some PTH. Thus, examining the *Gcm2* nulls and re-investigating the *Pth* nulls in the same genetic background (Black Swiss) as the *Hoxa3* null mice were previously evaluated, helped determine if genetic background could explain the differences in skeletal phenotypes observed between the *Pth* nulls (original report) and the *Hoxa3* nulls. Furthermore, it helped elucidate the relative roles of PTH, PTHrP, and the parathyroids in fetal calcium and addressed whether the *Hoxa3* null phenotype is due to the absence of PTH alone.

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III. Materials and Methods

A. Animal Husbandry

1. *Pth* Null Mice

The *Pth*^{tm1Dgo} knockout mice (i.e. *Pth* null mice) utilized in this study were obtained from Dr. Andrew Karaplis (McGill University, Montreal, Quebec, Canada), and were in a C57BL/6 background. Targeted ablation of the murine *Pth* gene, specifically replacing the coding sequence of mature PTH on exon 3 with a neomycin (NEO) resistant gene, was achieved by homologous recombination in embryonic stem cells as previously described (73).

2. *Gcm2* Null Mice

The *Gcm2*^{tm1Kry} knockout mice (i.e. *Gcm2* null mice) utilized in this study were obtained from Dr. Gerard Karsenty at the Baylor College of Medicine, Houston, TX, USA (now at Columbia University, New York, NY, USA), and were in a C57BL/6 background. As previously described, the knockout mice were generated by using embryonic stem cell technology and all four exons were replaced with a NEO cassette (79).

3. Colony Maintenance and Backcrossing

Both the *Pth* and *Gcm2* mice were back crossed into Black Swiss (Taconic Farms Inc., Germantown, NY), an outbred strain of mice, for at least three generations prior to

the start of any studies and then almost annually so the mice were closer to 7-10 generations in the latter experiments. Colonies were then maintained by mating heterozygous-deleted *Pth* males and females, and *Gcm2* males and females to produce litters that contained wild-type (WT or +/+), heterozygous (+/- or HET), and (*Pth* or *Gcm2*) null (-/-) mice.

4. Animal Housing

Animals were maintained in a facility operated by Animal Care Services of Memorial University of Newfoundland, in accordance with the Canadian Council on Animal Care (CCAC). The light/dark cycle consisted of 9:00 am – 9:00 pm of light, and 9:00 pm – 9:00 am of darkness. Water and standard mouse chow (1% calcium, 0.75% phosphorus) was give *ad libitum*. Male breeders were housed individually, while females were housed 4 per cage. All experimental procedures were approved by the Institutional Animal Care Committee (IACC) of Memorial University of Newfoundland.

5. Timed Matings

Pth +/- males and females and *Gcm2* +/- males and females were mated overnight twice weekly. The following morning, the females were checked manually for the presence of a vaginal mucous plug. The presence of a plug marked embryonic day (ED) 0.5, where the normal gestational period in these mice is nineteen days. Non-plugged females were then returned to their original housing cage, until the next mating.

B. Genotyping of Animals

1. Mouse Identification

At approximately three weeks of age (the time of weaning) mice were briefly anaesthetized with Isoflurane™ (Baxter) and given a numbered ear tag for identification purposes.

2. Tail Clipping

At the time of weaning and when fetal samples were collected, a small portion of tail was clipped from each of the mice. The piece of tail was then placed in a 1.5 ml microcentrifuge tube (Fisher Scientific) that contained 500 µl of lysis buffer (100 mM Tris-hydrogen chloride [HCL], pH 8.0 / 500 mM ethylenediaminetetraacetic acid [EDTA], pH 8.0 / 0.2% sodium dodecyl sulfate [SDS] / 200 mM sodium chloride [NaCl], and 100 µg/ml of proteinase K [Invitrogen]). The tubes were then placed in an isotherm oven (Fisher Scientific) at 55°C for 18-24 hours. Following this incubation period the tail samples were digested and ready for deoxyribonucleic acid (DNA) extraction.

3. DNA Extraction

Tail samples were removed from the oven, shaken by hand for 2-3 minutes and then centrifuge at 13, 000 rpm for 10 minutes. The supernatant was then transferred to fresh a microcentrifuge tube containing 0.5 ml of isopropanol, and were inverted several times to precipitate the DNA. Small pipette tips were then used to transfer the

precipitated DNA into a microcentrifuge tube containing 0.5 ml of deionized water. Samples were then shaken by hand for 5 minutes to dissolve the DNA, and 0.5 ml of phenol/chloroform/isoamyl alcohol (100:100:1) was added. Next, the samples were shaken vigorously for 1 minute and then centrifuged for 2 minutes at 13, 000 rpm. The aqueous layer was then transferred to a fresh microcentrifuge tube by pipetting, and 1.0 ml of cold 0.12 M sodium acetate (NaOAc) in ethanol (EtOH) was added. Tubes were inverted several times and then centrifuged for 10 minutes at 13, 000 rpm to form a DNA pellet. The supernatant was then discarded, and 1.0 ml of 70 % EtOH was added. After centrifuging for another 10 minutes, the EtOH was decanted, and any remaining EtOH was removed by pipetting. The DNA pellet was then air dried, resuspended in Tris EDTA buffer (TE) and stored at 4°C until samples were genotyped (by Polymerase Chain Reaction).

4. Polymerase Chain Reaction (PCR)

To genotype the mice, PCR was carried out on the DNA that was extracted from the mouse tails. For both the *Pth* and *Gcm2* colonies a four primer system was utilized. That is, two primers were utilized to detect the normal gene (i.e. *Gcm2* or *Pth* gene) and two primers were utilized to detect the presence of the NEO cassette.

The normal WT *Gcm2* gene was detected by utilizing the two sequence specific primers below:

G-F (forward): 5' - CAC CCT GCC AAA TGG TGG ATA ACA (Invitrogen)

G-B (reverse): 5' - CCC TCG CAT AAG TCA GCA AAC ATC (Invitrogen)

The normal WT *Pth* gene was detected by utilizing the two sequence specific primers below:

PTH1 (forward): 5'- GAG GCT TTG TAG TGG GTT TT (Invitrogen)

PTH2 (reverse): 5'- AGA GAA GTG GAA ATG GAG AG (Invitrogen)

The *Pth* null and *Gcm2* null were detected by utilizing two specific primers for the neomycin (NEO) resistant gene:

N1 (forward): 5' - GGA GAG GCT ATT CGG CTA TGA C (Invitrogen)

N2 (reverse): 5' - CGC ATT CGA TCA GCC ATG ATG G (Invitrogen)

The PCR cocktail included 10X PCR buffer (20 mM Tris-HCL, pH 8.4, 50 mM potassium chloride (KCL) [Invitrogen]), deoxynucleotide triphosphates (dNTP's) (0.2 mM of each deoxycytidine triphosphate (dCTP), deoxyguanosine triphosphate (dGTP), deoxyadenosine triphosphate (dATP) and deoxythymidine triphosphate (dTTP) [Invitrogen]), the appropriate primers (PTH1, PTH2 or G-F, G-B and N1 and N2 [Invitrogen]), 50 mM magnesium chloride ($MgCl_2$) [Invitrogen], Taq DNA polymerase (0.02 U/µl [Invitrogen]) and deionized water. 49 µl of the cocktail was then added to PCR tubes (Fisher Scientific) and 1.5 µl of the DNA sample obtained from the mouse tail was added. PCR tubes were then placed in the Peltier Thermal Cycler (PT-200 DNA

Engine Thermal Cycler - Dual Alpha Blocks) where the following PCR conditions were applied.

For the *Pth* primers: denaturation at 94°C for 45 seconds, annealing at 59°C for 40 seconds and then elongation at 72°C for one minute. These steps were repeated for 35 cycles, and were followed by 7 minutes at 72°C. Samples were then held at 4°C forever.

For the *Gcm2* primers: denaturation at 94°C for 45 seconds, annealing at 62°C for one minute, followed by elongation for one minute at 72°C. After repeating these steps 34 times, there was 10 minutes at 72°C. Samples were then held at 4°C.

5. Gel Electrophoresis

The PCR products were then electrophoresed on a 1.2 % agarose gel (1.2 g of agarose [Invitrogen] / 10 ml of 10X TAE [pH 8.0, 0.12 M EDTA, 0.40 M Tris, 11.5 % Glacial Acetic Acid] / 90 ml of deionized water / 0.06 % of Ethidium Bromide [Invitrogen] or 0.20 % of SYBR Safe™ Stain [Invitrogen]) immersed in 700 ml of 1X TAE buffer. 2 µl of Orange G loading dye (10 ml of 10X TAE / 500 mg of Orange G powder / 50 ml of glycerol) was added to the PCR tube following completion of PCR. 10 µl of each PCR sample was then loaded into the wells of the agarose gel and run for approximately 30 minutes at 200 volts in an EASY-CAST™ electrophoresis system. A picture of the gel was then taken using a Kodak GEL LOGIC 100 Imaging System with Kodak Molecular Imaging Software. For the *Pth* colony, the presence of a single 520 base pair (bp) band indicated that the mouse was WT for the *Pth* gene, the presence of a single 300 bp band indicated that the mouse was null for *Pth* gene, while the presence of

both bands indicated that the mouse was +/- for the *Pth* gene (**Figure 8**). For the *Gcm2* colony, the WT band was denoted by a single 572 bp band, the *Gcm2* null was denoted by a single 300 bp band, while the *Gcm2* +/- mice showed the presence of both bands (**Figure 9**).

C. Blood, Amniotic Fluid and Tissue Collection

1. Blood Collection

On embryonic day (ED) 18.5 heterozygous maternal whole blood was collected from the tail vein. The fetuses were quickly removed by cesarean section (C-section) and the fetal whole blood was collected after carotid/jugular transection. Maternal and fetal whole blood was collected in 60 µl heparinized capillary tubes (Bayer) and kept on ice until ionized calcium was measured.

For serum collection, both the maternal and fetal whole blood samples were collected in 60 µl micro-hematocrit capillary tubes (Bayer). Capillary tubes containing the blood were then placed in a Micro-MB microcentrifuge (Thermo Scientific) equipped with the capillary tube plate rotor. Samples were spun for 5-10 minutes at 14, 000 rpm. When samples were removed, the serum was clearly separated from the clotted blood. A glass cutter was then utilized to cut the capillary tube at the junction where the serum and clotted blood met. The serum was then decanted into 0.6 ml microcentrifuge tubes (Fisher Scientific) and stored at – 20 °C.

For plasma collection, 60 µl micro-hematocrit capillary tubes were flushed with 40 TIU/ml aprotinin (Sigma) in 0.5 M EDTA and used to collect the maternal and fetal whole blood samples. The samples were then decanted into 0.6 ml microcentrifuge tubes containing 2 µL of 40 TIU/ml aprotinin. Fresh capillary tubes were again flushed with aprotinin and then used to collect the samples from the microcentrifuge tubes. To separate the plasma, samples were then centrifuged for 5-10 minutes at 14, 000 rpm. Plasma was collected from the capillary tubes, placed in 0.6 ml microcentrifuge tubes and stored at – 70 °C.

Following blood collection, whole fetuses were then weighed and stored at – 20 °C for ashing.

2. Amniotic Fluid Collection

On ED 17.5 or 18.5 a terminal C-section, by cervical dislocation, was carried out on pregnant heterozygous females and the intact uterus was removed. Each amniotic sac was then lanced and the fluid was collected in 60 µl micro-hematocrit capillary tubes. Samples were transferred to 0.6 ml microcentrifuge tubes and stored at – 20 °C.

3. Whole Fetus and Tissue Collection

On ED 18.5 terminal C-section were carried out on heterozygous females. Whole fetuses and/or tissue samples were then collected and stored appropriately depending on the future use of the specimen.

Intact fetuses that were to be utilized for histology were placed in 10% buffered formalin (37% formaldehyde / 10X PBS [pH 7.3, 1.37 M NaCl, 0.027 M KCl, 0.43 M $Na_2HPO_4 \bullet 7H_2O$, 0.015 M KH_2PO_4, deionized water] / distilled water). From theses samples, fetal tibias were dissected from the whole fetus and embedded in paraffin. 5μm sections were cut using a Leica RM 2135 microtome.

Intact fetuses used for Alizarin Red S and Alcian Blue staining were genotyped by removing a portion of the skin, rather than the tail, and no blood samples were taken. Whole fetuses were then immediately placed in 95% EtOH. At that point, they were ready to start the staining protocol.

At the time of terminal C-sections, whole placentas and neck samples were also collected from some fetuses. The neck samples were made by coronal cuts just below the fetal mandible and above the sternal notch in order to include the thyroid and parathyroids. Ribonucleic acid (RNA) was to be extracted from these samples, thus they were immediately snap-frozen using liquid nitrogen, and samples were stored at – 70 °C in RNase/DNase free 1.5 ml microcentrifuge tubes.

D. Placental Calcium Transfer

On ED 17.5 or 18.5 pregnant heterozygous females were briefly anesthetized with Isoflurane™ and administered a 100 μl intracardiac injection consisting of 50 μCi of ^{45}Ca and 50 μCi of ^{51}Cr-EDTA in 0.9% saline. Females were then placed in a plexiglass box coated in lead for 5 minutes, after which time a terminal C-section was carried out by cervical dislocation. The uterus was quickly removed and then each fetus was removed

from its amniotic sacs. The fetuses were placed in 12 mm × 75 mm disposable round bottom polystyrene culture test tubes (Fisher Scientific). Forceps were used to pith the brain and the tubes were then capped. The ^{51}Cr-EDTA activity in each fetus was then determined using a 1480 WIZARD™ 3" automatic gamma counter (Perkin Elmer). 1 ml of Scintigest™ (Fisher Scientific) was then added to each tube and vortexed until the fetuses began to spin. Fetuses (with the 1 ml of Scintigest™) were then transferred to a disposable 20 ml scintillation vial (Fisher Scientific). 9 ml of Scintigest™ was then added to each of the vials, which were then placed in an isotherm oven (Fisher Scientific) at 55°C for 24 to 48 hours. The fetuses were vortexed several times during this time. When the fetuses were fully solubilized they were removed from the oven, and 10 ml of ScintiVerse® (Fisher Scientific) and 5 drops of glacial acetic acid (Fisher Scientific) was added to each vial. The vials were then completely covered in aluminum foil and placed in the dark for 24 hours to reduce bioluminescence. The activity of ^{45}Ca present in each fetus was then determined using a LS 6500 Multi-Purpose Scintillation Counter (Beckman Coulter). After the activity of each isotope present in each fetus was determined, the ratio of ^{45}Ca to ^{51}Cr-EDTA activity for each fetus was used to determine the calcium transfer. The ratios were then normalized to the mean value of the heterozygotes within each litter [i.e. percent of the HET mean (% of +/- Mean)]. Heterozygotes were chosen to be the baseline comparison because heterozygote fetuses account for approximately 50% of fetuses in a litter and therefore provided a more robust and stable value for normalization. Placental calcium transfer measurements in relevant

previous studies have been reported in this manner (67, 72). Normalization allowed all the litters to be combined for analyses.

E. Fetal PTH Injections (*in utero*)

On ED 18.5, *Pth* +/- dams were anesthetized by administering an intraperitoneal injection of Pentothal® (Abbott Laboratories, Limited). A C-section was then carried out on the mother and approximately half of the fetal mice were injected *in utero* with 1 nmol PTH [1-84] (Bachem) in 2μl of saline, while the other half were injected with 2 μl of saline. The intraabdominal injections were administered to the left side of each fetus (to avoid puncturing the liver) using a microinjection pipette and sutures were placed over the gestational sacs of the PTH injected fetuses so they could be identified later. The mother's incision was then closed, and she was permitted to awaken. To determine the effect of exogenous PTH on placental calcium transfer, 85 minutes after the fetal injections, the placental calcium transfer procedure was performed. Thus, placental calcium transfer was assessed a total of 90 minutes after the fetal injections. This time point was chosen because it was consistent with the previously discussed study which found that placental calcium transfer in the *Pthrp* null fetuses increased when assessed 90 minutes following a fetal injection of PTHrP [1-86] or PTHrP [67-86] (67). To determine the effect of the PTH *in utero* injections on gene expression in the placenta, a terminal C-section (also carried out 90 minutes following the fetal injections) was carried out and the placentas were collected and snap-frozen in liquid nitrogen. They were stored at – 70 °C for later RNA extraction and microarray analysis.

The placental calcium transfer methodology utilized in this study requires fetuses from approximately 6-10 pregnant mice to compare the baseline rate of placental calcium transfer among WT, +/- and null fetuses. In order to compare the effect of a single treatment versus saline at one time point, the number of mice required more than doubles. Furthermore, as the procedure is terminal, measurements can only be done at a single time point after the *in utero* injection. Consequently, it was not feasible to study a time-course of PTH injections or determine a full dose-response curve. Thus, the 1 nmol of PTH was chosen because it was equimolar to the dose of PTHrP shown in previous studies to be effective in increasing the rate of calcium transfer in the *Pthrp* null mice (67). However, if the 1 nmol of PTH had not worked, before moving on to another dose, it would have been necessary to confirm that the PTH utilized in the study was biologically active. This would have been accomplished by evaluating the cAMP response in cultured cells to the PTH [1-84]. But since a response was seen, I did not think it was necessary to do this afterwards.

F. RNA Extraction

RNA was extracted using the RNeasy Midi Kit (Quiagen). RNA was quantified by UV spectrophotometry, while RNA quality was assessed by utilizing both UV spectrophotometry measurements and visually observing the ribosomal RNA band integrity by electrophoresing the samples on an agarose gel. The Agilent 2100 BioAnalyzer (Agilent Technologies, Inc.) was used to determine the RNA quantity and quality of the samples utilized for microarray analysis.

G. Complementary DNA (cDNA) Synthesis

cDNA synthesis was performed using the SuperScript III First-Strand Synthesis System and carried out as per the product insert.

H. Real-Time Quantitative Reverse Transcriptase-PCR (Real-Time Quantitative RT-PCR)

TaqMan® Gene Expression Assays for S100g (i.e. CaBP9k), VDR, PTHrP, PTH and TRPV6 were utilized in the present study. These assays are pre-designed and pre-optimized primers and probes with a fluorescent reporter dye (FAM™) at the 5' end of the TaqMan® MGB probe and a non-fluorescent quencher at the 3' end. In addition to the TaqMan® Gene Expression Assays, TaqMan® Universal PCR Master Mix (Applied Biosystems) was used when PTHrP expression was assessed in both neck and placenta RNA samples. The thermal cycler protocol then consisted of a 2 minute cycle at 50 °C, a 10 minute cycle at 95 °C, followed by 40 cycles of 15 seconds at 95 °C and 1 minute at 60 °C. When CaBP9k, VDR, PTH and TRPV6 expression was assessed in placenta samples however, in addition to the TaqMan® Gene Expression Assays, the TaqMan® RNA-to- C_T™ *1-Step* Kit (Applied Biosystems) was used instead of the Universal PCR Master Mix. This eliminated the need to carry out a separate cDNA synthesis step prior to quantitative real-time RT-PCR. When the TaqMan® RNA-to- C_T™ *1-Step* Kit was used the thermal cycler protocol then consisted of a 15 min cycle at 48 °C, a 10 minute cycle at 95 °C, followed by 40 cycles of 15 seconds at 95 °C and 1 minute at 60 °C. All real-time quantitative RT-PCR was carried out in the ABI PRISM 7000 Sequence Detection

System (Applied Biosystems). All samples were performed in triplicate with a reaction volume of 20µl. GAPDH was used as a control gene, and the gene expression ratios were then calculated from differences in the threshold cycle (C_T).

I. Skeletal Morphology and Mineral Assessment

1. Whole Blood Ionized Calcium

Maternal whole blood which was collected from the tail vein, and fetal whole blood which was collected by carotid/jugular transection, were kept on ice until analyzed within 30 minutes using a Chiron Diagnostics 634 Calcium/pH Analyzer (Ciba Corning Diagnostics Limited).

2. Serum and Amniotic Fluid Magnesium and Phosphorus

Magnesium and phosphorous in the serum and amniotic fluid were measured using colorimetric assays in the *Gcm2* colony (Sigma-Aldrich). These kits were discontinued and this necessitated that different colorimetric assays be used in the *Pth* colony (Diagnostic Chemicals Limited, Charlottetown, PEI). All assays were carried out as per the product insert.

3. Alizarin Red S and Alcian Blue Staining

The day after whole fetuses (ED 18.5) were collected and placed in a scintillation vial containing 95 % EtOH, the skin was carefully peeled off. Fetuses were then

transferred to acetone to remove the fat. Once the fat was removed, the fetuses were then covered in 15 ml of staining solution (0.3 % alcian blue 8GS in 70 % EtOH [1 volume]/ 0.1 % alizarin red S in 95 % EtOH [1 volume]/ acetic acid [1 volume]/ 70 % EtOH [17 volumes]) for 3 days at 37 °C. The staining solution was then aspirated off and the fetuses were washed in distilled water. Fetuses were then placed in 15 ml of a 1 % aqueous solution of potassium hydroxide (KOH). Fetuses were checked daily, and when the skeleton was clearly visible through the surrounding tissue, the fetuses were cleared through a series of 20 %, 50 % and 80 % glycerol/ 1 % aqueous KOH solutions over a 2 to 4 week period. After the fetuses were fully cleared, they were transferred to 100 % glycerin and stored at room temperature.

4. Von Kossa Staining

Paraffin embedded fetal (ED 18.5) tibia sections were placed on a slide warmer (Fisher) for 30 minutes and then put through the following steps to deparafinize and rehydrate the samples: Xylene – 2 minutes (2x), 100 % EtOH – 2 minutes (2x), 95 % EtOH – 2 minutes, 70 % EtOH– 2 minutes, 50 % EtOH – 2 minutes, distilled water – 1 minute. Sections were then placed in a 1 % aqueous silver nitrate solution and placed under a strong light for 60 minutes. Following exposure to the silver nitrate, the sections were washed in distilled water 3 times for 2 minutes each time. Sections were then transferred to a 2.5 % sodium thiosulphate solution for 5 minutes, and then washed three times (2 minutes each time) in distilled water. Sections were then counterstained with methyl green (Sigma) for 2 minutes. Excess dye was blotted from the slides, washed in 1-

butanol (Fisher Scientific) twice (10 seconds each time) and then washed in xylene twice (10 seconds each time). Permount (Fisher Scientific) was then added to the slides to mount the coverslips.

5. Fetal Ash Weight and Flame Atomic Absorption Spectroscopy

Fetuses that were previously genotyped were weighed, placed in weighed crucibles and reduced to ash in an oven at 500 °C for 24 hours. After the fetuses were reduced to ash, the crucible was once again weighed to determine the amount of ash present. The ash weight reflects the amount of total mineral present in the fetal skeleton. To control for fetal size, which varies from litter to litter, ash weight data was normalized to the mean value of the WT fetuses within each litter [i.e. percent of the WT mean (% of WT Mean)].

All ash samples were transferred to 20 ml scintillation vials and stored at room temperature until the samples were prepared for Atomic Absorption Spectroscopy analysis to determine the amount of calcium and magnesium present in the skeletal ash. To do this, 253 µl of nitric acid was added to each scintillation vial containing the fetal ash. Samples were left to sit for at least 5 days at room temperature for the ash to dissolve, and then 10 ml of deionized water was added to each vial. Appropriate dilutions were made for the reading to be within the proper calibration range of the 2380 Atomic Absorption Spectrophotometer (Perkin-Elmer). Like ash weight data, skeletal mineral content data was also normalized to the mean value of the WT fetuses within each litter (% of WT Mean).

J. Hormone Assays

1. PTH Enzyme-Linked ImmunoSorbent Assay (ELISA)

Serum PTH levels were determined using a Rat Intact PTH [1-84] ELISA Kit (Immutopics). The procedure outlined in the kit was followed and the absorbance of each sample was determined using a Vmax Kinetic Microplate Reader (Molecular Devices). The detection limit of the kit was 1.6 pg/ml and samples with a concentration below this limit were assigned a value equal to the detection limit.

2. PTHrP Radioimmunoassay (RIA)

Plasma samples were sent to the St. Vincent's Institute of Medical Research, Melbourne, Australia, for PTHrP analysis. The RIA was designed by Dr. T. J. Martin, and utilized an antibody specific for the amino-terminal portion of PTHrP. All samples were analyzed in duplicate. A sufficient volume of plasma was collected from each adult mouse to constitute one sample, while fetal samples had to be pooled to obtain the necessary volume. See **Appendix C** for a detailed protocol.

In fetal mouse plasma, it is likely that the sensitivity of this assay is 3.8 ± 0.3 pmol/l. This is due to the fact that previous studies reported plasma PTHrP in *Pthrp* null fetuses to be 3.8 ± 0.3 pmol/l (72).

K. Affymetrix GeneChip® Analysis

RNA samples were shipped on dry ice to The Centre for Applied Genomics, Microarray Facility, at the Hospital for sick Children in Toronto, ON. This facility is an accredited Affymetrix service provider and initially carried out RNA quality testing via the Agilent 2100 BioAnalyzer (Agilent Technologies, Inc.). All samples were deemed to be of good quality, with RNA Integrity Numbers (RIN) ranging from 8.4 – 9.0 (see **Appendix D** for detailed bioanalyzer results). The Mouse Gene ST 1.0 Array, which contains numerous probes spread across the full length of every well-annotated gene, was then utilized to assess gene expression in the placenta of WT and *Pth* null fetuses that were injected with either saline or exogenous PTH [1-84]. In total, 12 arrays were completed, which included 3 samples in each of the following groups: WT injected with saline, *Pth* null injected with saline, WT injected with PTH [1-84], *Pth* null injected with PTH [1-84]. Raw data obtained from the Mouse Gene ST 1.0 Arrays, which summarizes all probes into a single gene expression value, were then sent to the Statistical Analysis Core Facility, also at The Centre for Applied Genomics, for analysis. At the Facility, gene annotation information was obtained from Affymetrix (March, 2008 version) and any probesets in the normalized data without gene names/gene assignments were removed. As a result, the final data set consisted of 22, 158 probesets. The raw data was initially normalized using the robust multi-array average (RAM) algorithm (116) and differentially expressed genes were then identified using the Local-Pooled-Error Test (LPE) (117). In this method, errors within genes and between replicate arrays for genes in which expression values are similar are pooled, and it has been shown to have an

advantage in identifying subtle changes in gene expression. False discovery rate (FDR) (118) was used to evaluate statistical significance and was controlled at 0.05, such that genes with adjusted p-values < 0.05 were considered to be statistically significant.

L. Statistical Analysis

For all experiments fetuses from several heterozygous mothers were used to avoid the bias of a single mother contributing to the data. All data was analyzed using SYSTAT 5.2.1 for Macintosh (SYSTAT, Inc., Evanston, IL). Analysis of variance (ANOVA) was initially used to analyze the data, followed by a post hoc test to determine significant differences between pairs of means. The real-time quantitative RT-PCR data was analyzed using the $2^{-\Delta\Delta C}T$ method (119). All data are presented as the mean ± standard error (SE). Two-tailed probabilities are reported, and a probability (p) less than 0.05 ($p < 0.05$) was considered to be statistically significant. $p > 0.05$ was considered to be not significant (NS) and is denoted by p = NS.

Details of the statistical analysis for the microarray can be found in the prior Affymetrix GeneChip® Analysis section (Section III. I.).

IV. Results

A. Serum PTH

As expected, the serum PTH was at or below the limit of detection of the assay (1.6 pg/ml) in all *Pth* null fetuses (**Figure 10A**). Serum PTH was also low in *Gcm2* null fetuses as compared to *Gcm2* +/- fetuses (**Figure 10B**). Although PTH was at or below the detection limit in some samples, detectable values ranged from 2.96 - 31.11 pg/ml.

The low serum PTH levels in the *Gcm2* null fetuses were quite surprising. The initial reports by Gunther *et al* indicated that the serum PTH levels in *Gcm2* nulls were normal (79). The reason for the large difference in serum PTH levels between the present and previous study is unknown, however, there are several factors that may be contributing to the different findings. First, in the present study the mice were studied in a Black Swiss background, while in the previous study the mice were maintained in the C57BL/6 background. Second, measurements in the present study were taken in fetal mice (ED 18.5), while adult mice were the subjects of the previous study. Finally, serum PTH levels in both studies were measured by using different methods. In the present study a rat intact PTH [1-84] ELISA kit was utilized, while in the former study an immunoradiometric assay specific for rat PTH [1-34] was used.

B. Blood and Serum Chemistries

The blood and serum chemistry of *Pth* nulls and *Gcm2* nulls was remarkably similar. *Pth* null and *Gcm2* null fetuses were equally hypocalcemic, with an ionized

calcium that was 25% lower than in the +/- and WT fetuses (**Figure 11A and 11B**). The ionized calcium in *Pth* nulls and *Gcm2* nulls was equal to the maternal level, with *Pth* +/- mothers at 1.21 ± 0.06 mmol/L (n=8) and *Gcm2* +/- mothers at 1.31 ± 0.03 (n=7). *Gcm2* +/- and *Pth* +/- fetuses did not show any reduction in ionized calcium. In both *Pth* null and *Gcm2* null fetuses serum phosphorous was significantly higher as compared to WT and +/- fetuses (**Figure 12A and 12B**). Serum magnesium in both *Pth* nulls and *Gcm2* nulls was reduced. In *Pth* nulls serum magnesium was no different as compared to *Pth* +/- fetuses (p=NS) (**Figure 13A**), while serum magnesium was slightly, but significantly reduced in *Gcm2* nulls (0.87 ± 0.01 mmol/L, n=11) as compared to *Gcm2* +/- fetuses (0.92 ± 0.01 mmol/L, n=19) (p=0.004) (**Figure 13B**). Although statistically significant, the decrease in serum magnesium observed in the *Gcm2* null is very small and may be physiologically unimportant.

Ionized calcium, serum phosphorous and serum magnesium levels between the *Pth* +/- mothers and *Gcm2* +/- mothers were no different (p=NS).

C. Amniotic Fluid Calcium and Magnesium

The amniotic fluid is comprised mainly of fetal urine and thus can be used to evaluate the amount of mineral that the fetal kidneys are excreting (120). From previous studies (unpublished from Dr. C.S. Kovacs laboratory) it has been shown that mineral amniotic fluid measurements do not differ significantly from ED 17.5 or 18.5. Thus, all amniotic fluid data in the current study is presented collectively. It was found that in both *Pth* null and *Gcm2* null fetuses amniotic fluid calcium (**Figure 14A and 14B**) and

magnesium (**Figure 15A and 15B**) were low as compared to +/- fetuses. This is consistent with both *Pth* nulls and *Gcm2* nulls having a reduced renal filtered load because they have lower serum levels of these minerals.

D. Skeletal Morphology and Mineralization

When examining whole fetal skeletons stained with alcian blue (for cartilage) and alizarin red (for mineralized tissue), it appeared that both *Pth* null (n=3) and *Gcm2* null (n=3) fetal mice were normal in size as compared to WT (n=3). Furthermore, the morphology of the skeleton and the mineralization pattern of both *Pth* nulls and *Gcm2* nulls appeared no different than that of WT siblings (**Figure 16 and 17**). That is, before birth, the skeletal scaffold had developed normally, with normal long bone and tibial diaphyseal lengths, and the appropriate areas of the fetal skeleton were mineralized. These findings are in contrast to the original *Pth* null studies which showed that *Pth* nulls were dysmorphic with (among other things) slightly shortened long bones, abnormally formed skulls, vertebral bodies, and shortened metacarpal and metatarsal bones (73). The sole difference between *Pth* nulls in the former study and *Pth* nulls in the present study is the genetic background: C57BL/6 in the former, and Black Swiss in the present.

When fetal tibia sections were examined in more detail, by Von Kossa staining, it was evident that the overall cellular morphology and growth plates were normal in both *Pth* null (n=6) and *Gcm2* null (n=6) fetuses as compared to WT (n=6) siblings. In addition, both *Pth* nulls and *Gcm2* nulls appeared to have no obvious changes in mineral deposition as represented by the black deposits due to von Kossa's stain (**Figure 18 and**

19). However, when the fetal skeletons were reduced to ash in order to assess the amount of total mineral present, the ash weight of both *Pth* null and *Gcm2* null fetuses was reduced by approximately 5% and 10% respectively, as compared to WT fetuses (**Figure 20A and 20B**). This is consistent with the previously published study which showed that skeletal mineralization in the *Pth* nulls was significantly less then that of WT mice (73). The fetal ash was examined by Atomic Absorption Spectroscopy, by which means it was determined that skeletal calcium content was reduced by 15% in the *Pth* nulls as compared to WT (**Figure 21A**), while it was reduced by 19% in the *Gcm2* null fetuses as compared to WT fetuses (**Figure 21B**). Skeletal magnesium content was also significantly reduced, by 18% in *Pth* nulls, and 14% in *Gcm2* nulls as compared to their respective WT fetuses (**Figure 22A and 22B**).

E. Placental Calcium Transfer

The amount of calcium transferred from the mother to the fetus in a 5 min period was measured to determine if a decrease in PTH or absence of PTH would affect the normal placental calcium transfer process. Previous findings (unpublished from Dr. C. S. Kovacs laboratory) indicate that calcium transfer occurs rapidly on both ED 17.5 and 18.5, and that there is no significant difference between the two days. Thus, in the present study, all placental calcium transfer data is presented collectively. In the *Pth* null fetuses, placental calcium transfer was no different as compared to *Pth* +/- fetuses (**Figure 23A**). *Gcm2* nulls increased calcium transfer by approximately 20 % as compared to *Gcm2* +/- fetuses (**Figure 23B**). Thus, although *Pth* null and *Gcm2* null fetuses showed identical

biochemical phenotypes (hypocalcemia, hypomagnesemia, and hyperphosphatemia), the *Gcm2* nulls displayed increased placental calcium transfer. The increase in placental calcium transfer does not obviously affect the serum biochemistry of the *Gcm2* nulls. I speculate that the increased rate of transfer enables the *Gcm2* nulls to maintain a blood calcium level at the maternal level. In other words, if the aparathyroid *Gcm2* nulls hadn't increase placental calcium transfer, then the biochemical phenotype would have been more severe and similar to the aparathyroid *Hoxa3* nulls. Further investigations were done to determine why placental calcium transfer was upregulated in *Gcm2* nulls but not in the *Pth* nulls.

F. Plasma PTHrP

It has been previously reported that high plasma PTHrP levels are associated with an increase in calcium transfer across the placenta (67, 72). Plasma PTHrP was measured to determine if increased plasma PTHrP could explain the increase in placental calcium transfer observed in the *Gcm2* null mice. The results obtained from the PTHrP RIA showed that plasma PTHrP in both *Pth* null and *Gcm2* null fetuses was no different as compared to their respective WT and HET littermates (**Figure 24A and 24B**). Thus, circulating PTHrP may not be responsible for the increase in placental calcium transfer observed in the *Gcm2* null fetuses, and PTHrP does not upregulate in response to hypocalcemia, a finding consistent with what was previously observed in *Hoxa3* nulls (72) (**Table 1**). Plasma PTHrP was also not significantly different between *Pth* +/- mothers (n=6) and *Gcm2* +/- mothers (n=6) (p=0.97).

Using the same PTHrP assay utilized in the present study, in previous studies it was reported that *Pthrp* null fetuses had a plasma PTHrP value of 3.8 pmol/l. Because *Pthrp* null fetuses cannot make PTHrP, and since PTHrP does not cross the placenta, the value of 3.8 pmol/l likely represents the sensitivity (detection limit) of this assay in fetal mice. It has also been shown that WT fetuses had plasma PTHrP levels that were typically 6-8 pmol/l (same as the WT values in the present study), and that plasma PTHrP in *Pthr1* null fetuses was 63.5 pmol/l. Thus, the assay can clearly distinguish between low (3.8 pmol/l), normal (6-8 pmol/l) and markedly high (63.5 pmol/l) PTHrP levels (72). Consequently, it is likely that the lack of change in PTHrP levels in the *Gcm2* nulls and *Pth* nulls as compared to WT and HET fetuses is real and not due to failure of the assay to distinguish a difference .

G. PTHrP Expression in the Neck and Placenta

Although plasma PTHrP levels were no different between genotypes in either the *Pth* colony or the *Gcm2* colony, knowing that it is controversial whether the fetal parathyroids express PTHrP or not we wanted to rule out the possibility that PTHrP was upregulated in the parathyroids. Thus, the expression of PTHrP in the neck region (which would include the parathyroid glands) was assessed by quantitative real-time RT-PCR. As previously mentioned, PTHrP is expressed in many fetal tissues, thus it is not surprising that PTHrP was detected in the fetal neck sections. As can be seen in **Figure 25A and 25B**, the expression of PTHrP messenger RNA (mRNA) in the neck of both *Pth* null and *Gcm2* null mice was no different than that of WT mice. Like the plasma PTHrP

results, this supports the idea that PTHrP may not be responsible for the increase in the transfer of calcium observed in the *Gcm2* null fetuses, and that PTHrP does not increase in response to fetal hypocalcemia.

PTHrP not only works in an endocrine manner, but also in an autocrine/paracrine manner in certain tissues. Thus, it is possible that PTHrP may not be increased in the plasma, but might be increased in the placenta and working within the trophoblasts or IPYS to increase the transfer of calcium from the mother to fetus. To determine if high placental PTHrP levels could account for the increase in calcium transfer observed in the *Gcm2* null fetuses, the expression of PTHrP in the whole placenta was assessed by quantitative real-time RT-PCR. As can be seen in **Figure 26A and 26B**, PTHrP mRNA expression in the placenta was no different between WT and *Pth* or *Gcm2* null fetuses. This further supports the notion that PTHrP is not responsible for the increase in calcium transfer observed in the *Gcm2* null fetal mice: it was not increased in plasma, and the mRNA was not increased in placenta or neck region. Also, it reaffirms that PTHrP does not upregulate in response to lack of PTH and fetal hypocalcemia.

H. Effect of Exogenous PTH Administration on Placental Calcium Transfer

The data obtained thus far show that plasma PTHrP and the expression of PTHrP mRNA in both the placenta and the neck of *Pth* null and *Gcm2* null fetuses is normal, both the *Gcm2* null and *Pth* null fetuses experience the same degree of hypocalcemia, but only the *Gcm2* nulls (which produce some PTH) upregulated placental calcium transfer. These led to the question whether PTH may play a role in regulating placental calcium

transfer, and if it could be a contributing factor to the increase in calcium transfer observed in the *Gcm2* null fetuses. *Gcm2* nulls have some circulating PTH while *Pth* nulls do not. To directly test the hypothesis that PTH plays a role in regulating placental calcium transfer, *Pth* null fetuses were injected *in utero* with either saline or 1.0 nmol of PTH [1-84]. The 1 nmol of PTH was chosen because it was equimolar to the dose of PTHrP that was administered and effective in increasing the rate of placental calcium transfer in *Pthrp* null mice (67), and the *Pth* null fetuses were utilized because they are the model that completely lacks PTH. Also, they do not have other abnormalities, such as lacking the parathyroids, thyroid or thymus. The presence of these other abnormalities (such as in *Hoxa3* nulls) may lead to confounding results.

As can be seen in **Figure 27**, *Pth* null fetuses did increase calcium transfer in response to injections of PTH [1-84] as compared to saline injections ($p<0.033$). WT and HET fetuses showed non-significant increases as well. This further supports the theory that PTH can stimulate placental calcium transfer. This increase in placental calcium transfer cannot be attributed to an alteration in ionized calcium because the ionized calcium levels did not differ between *Pth* null fetuses injected *in utero* with PTH [1-84] and *Pth* null fetuses injected *in utero* with saline (**Figure 28**).

I. Gene Expression in WT and *Pth* null Placenta treated *in utero* with either Saline or PTH [1-84]

To determine potential genes that may be contributing to the upregulation of placental calcium transfer observed in the *Pth* null fetuses when treated *in utero* with

PTH [1-84], a Mouse Gene ST 1.0 Array was utilized to evaluate differential gene expression in the placenta. As can be seen in **Table 2**, the expression of some genes known to be involved in regulating placental calcium transfer including *Trpv6* (93) and *Cabp9k* (92) were significantly decreased in *Pth* null fetuses as compared to WT fetuses. More specifically, *Trpv6* expression in the *Pth* null placenta was decreased by 60% ($p<<0.001$) as compared to WT placenta, while *Cabp9k* expression was decreased by 40% ($p<<0.001$). The *Vdr* was also shown to be significantly decreased (by 20%, $p<<0.001$) along with several other genes that are known to be involved in vitamin D metabolism including the human group-specific component (*Gc*) (i.e. Vitamin D-binding protein), which is involved in vitamin D transport and storage (121). Furthermore, the expression of several genes which are involved in cation and solute transport were also significantly reduced (e.g. *Slc3a1*, *Slc22a2*).

Trpv6, *Cabp9k* and *Vdr* mRNA expression was also assessed by real-time quantitative RT-PCR and confirmed that the expression of all 3 genes was significantly lower in the *Pth* null placentas as compared to WT (**Figure 29A, 29B, and 29C**). Similarly, as assessed by real-time quantitative RT-PCR the *Gcm2* null placentas also had reduced expression of both *Trpv6* and *Cabp9k* as compared to WT placentas (**Figure 30A and 30B**). *Vdr* expression however, was found to be no different between the *Gcm2* null and WT placentas (**Figure 30C**).

Despite the short time interval (90 min) between the PTH fetal injections and the harvesting of placentas, microarray analysis also revealed that when *Pth* null fetuses were treated *in utero* with PTH [1-84], *Vdr* expression increased 1.8 fold ($p<<0.001$) as

compared to placentas collected from *Pth* null fetuses injected with saline. Furthermore, the expression of several solute carriers (e.g. *Slc39a8*, *Slc23a3*) also increased in response to the *in utero* treatment of PTH [1-84] (**Table 3**). Real-time quantitative RT-PCR confirmed that *Vdr* expression increased (1.5 fold) in response to PTH [1-84] treatment (**Figure 31**).

Because the microarray results showed that absence of PTH as well as PTH treatment affected placental gene expression, we wondered if placental gene expression differed between the *Pth* nulls and *Gcm2* nulls. Despite the *Gcm2* null placentas also having significantly reduced expression of both *Trpv6* and *Cabp9k* as compared to WT and normal *Vdr* expression, real-time quantitative RT-PCR showed that the expression of *Trpv6*, *Cabp9k* and *Vdr* was significantly higher in the *Gcm2* null placenta as compared to placentas harvested from *Pth* nulls (**Figure 32A, 32B, and 32C**). This may explain the upregulation of placental calcium transfer observed in the *Gcm2* null fetuses.

It should be noted that the difference in VDR gene expression in the *Pth* null placenta as compared to the *Gcm2* null placenta (**Figure 32C**) may appear to be exaggerated when comparing it to the figures that show the difference in placental VDR expression in the *Pth* nulls versus WT (**Figure 29C**) and *Gcm2* nulls versus WT (**Figure 30C**). However, the data are not directly comparable. For the PTH colony (**Figure 29C**) all fetuses in the analysis were injected *in utero* with saline, while this was not the case for the *Gcm2* colony data. Furthermore, when the direct comparison was made between the *Pth* nulls versus *Gcm2* nulls (**Figure 32C**), neither of the nulls were treated *in utero*

with saline, and all samples were run on the same plate for real-time quantitative RT-PCR analysis.

J. Placental Expression of PTH

The results have shown that absence of PTH alters placental gene expression and that treatment with PTH causes opposing changes in some of these same genes. This prompted the consideration that if PTH is expressed in the placenta, and acts locally, that might explain how *Gcm2* null placentas upregulated *Trpv6*, *Cabp9k*, and *Vdr* expression and placental calcium transfer. As determined by quantitative real-time RT-PCR, *Pth* mRNA was detected in WT and *Pth* null and *Gcm2* null placenta. *Pth* expression was significantly lower in *Pth* null placentas as compared to WT **(Figure 33A)**, and did not appear until the 31st cycle. This means that *Pth* null placentas either lacked PTH completely (false positive detection) or had a small amount of PTH due to the presence of maternal cells in the placenta. Conversely, *Pth* expression was 1.2-fold higher (p=NS) in *Gcm2* null placentas compared to WT **(Figure 33B)**. Furthermore, as with *Trpv6*, *Cabp9k*, and *Vdr*, it was found that the expression of *Pth* mRNA in the *Gcm2* null placentas was significantly higher than that found in *Pth* null placentas. In fact, *Pth* expression was almost 7-fold higher in *Gcm2* null placentas than in *Pth* null placentas ($p<<0.001$) **(Figure 32D)**.

Table 1: Summary of previously published literature showing the effects of loss of parathyroids and PTH (*Hoxa3* null), loss of PTH (*Pth* null), loss of PTHrP (*Pthrp* null), and loss of the amino-terminal effects of PTH and PTHrP (*Pthr1* null) on fetal blood calcium, skeletal development and mineralization, placental calcium transfer, serum PTH and plasma PTHrP as assessed in late gestation

	Ionized calcium	**Skeletal Mineralization**	**Skeletal Scaffold Development**	**Placental Calcium Transfer**	**Serum PTH**	**Plasma PTHrP**
***Hoxa3* null**	Below maternal level (72)	Reduced (29)	Normal (29)	Normal (72)	Undetectable (72)	Normal (72)
***Pth* null**	?	Reduced (73)	Modestly abnormal (i.e. Slightly shortened long bones, shorter metacarpals and metatarsals) (73)	?	Undetectable (73)	?
***Pthrp* null**	At maternal level (67)	Normal (29)	Abnormal (i.e. Smaller overall, shortened limbs, rounded skull, shortened and disorganized growth plate, accelerated endochondral bone development) (29)	Reduced (67)	Increased (29)	Low (72)
***Pthr1* null**	Well below maternal level (67)	Reduced (29)	Abnormal (i.e. Similar to *Pthrp* null, but more severe) (29)	Increased (67)	Increased (29)	Increased (72)
***Gcm2* null**	?	?	?	?	?	?

Numbers in parentheses indicate citations
? = unknown

Table 2. Selected list of genes showing statistically significant differential regulation at baseline (i.e. saline injection) between *Pth* null versus WT placentas.

Gene	Gene Name	Fold Change	Adjusted p- value
Fabp1	fatty acid binding protein 1	2.11	0.013
Hbb-y	hemoglobin Y, beta-like embryonic chain	1.88	0.001
Krt1	Keratin 1	1.54	0.049
Atp7b	ATPase, Cu^{++} transporting, beta polypeptide	0.96	0.014
Osbpl6	oxysterol binding protein-like 6	0.84	0.002
Afp	alpha fetoprotein	0.84	<0.001
Lrp2 (megalin)	low density lipoprotein receptor-related protein 2	0.83	<<0.001
Dab2	disabled homolog 2 (Drosophila)	0.82	<<0.001
8430408G22Rik	RIKEN cDNA 8430408G22 gene	0.82	0.044
Slc27a2	solute carrier family 27 (fatty acid transporter)	0.81	<<0.001
Ahsg	Alpha-2-HS-glycoprptein	0.81	<0.001
* Vdr	vitamin D receptor	0.81	<<0.001
Apoc1	apolipoprotein C-I	0.80	0.019
Heph	hephaestin	0.80	<<0.001
Abcc2	ATP-binding cassette	0.79	<0.001
Slc13a3	solute carrier family 13 (sodium-dependent dicarboxylate transporter)	0.76	0.016
Slc7a9	solute carrier family 7 (cationic amino acid transporter	0.76	<<0.001
Apoa2	apolipoprotein A-II	0.76	<<0.001
Cubn	cubilin (intrinsic factor-cobalamin receptor)	0.75	<<0.001
2010003K11Rik	RIKEN cDNA 2010003K11 gene	0.75	0.002
Apom	apolipoprotein M	0.75	<<0.001
Trf	transferrin	0.75	<<0.001
5033414D02Rik	RIKEN cDNA 5033414D02 gene	0.74	<0.001
Slc22a2	solute carrier family 22 (organic cation transporter)	0.73	0.001
Apoc2	apolipoprotein C-II	0.72	<<0.001
Apob	apolipoprotein B	0.72	<<0.001
Rbp2	retinol binding protein 2	0.71	<<0.001
Ambp	alpha 1 microglobulin	0.70	<0.001
Slc6a19	solute carrier family 6 (neurotransmitter transporter)	0.69	<0.001
Slc5a1	solute carrier family 5 (sodium/glucose cotransporter)	0.69	<<0.001
Apoa1	apolipoprotein A-I	0.68	<<0.001

Gc (DBP)	group specific component (vitamin D binding protein)	0.67	<<0.001
Sfpd	surfactant associated protein D	0.65	0.003
Slc3a1	solute carrier family 3	0.64	<<0.001
Apoa4	apolipoprotein A-IV	0.64	<<0.001
* S100g (CaBP-D9K)	S100 calcium binding protein G (calbindin D9k)	0.58	<<0.001
Aqp8	aquaporin 8	0.46	<<0.001
* Trpv6	transient receptor potential cation channel, subfamily V, member 6	0.40	<<0.001

Table 3. Selected list of genes showing differential regulation at 90 minutes between PTH 1-84-injected versus saline-injected *Pth* null placentas.

Gene	Gene Name	Fold Change	Adjusted p- value
2010109I03Rik	RIKEN cDNA 2010109I03 gene	3.68	<<0.001
Slc39a8	solute carrier family 39 (metal ion transporter)	2.20	<<0.001
Sfpd	surfactant associated protein D	1.90	0.067
* Vdr	vitamin D receptor	1.79	<<0.001
Alb	albumin (Alb)	1.78	<0.001
2210415F13Rik	RIKEN cDNA 2210415F13 gene	1.55	<0.001
Slc23a3	solute carrier family 23 (nucleobase transporters)	1.43	0.083
Gc (DBP)	group specific component (vitamin D binding protein)	1.40	<<0.001
Apoa4	apolipoprotein A-IV	1.26	<<0.001
1300017J02Rik	RIKEN cDNA 1300017J02 gene	1.25	0.012
Rbp2	retinol binding protein 2	1.20	<0.001
Cubn	cubilin (intrinsic factor-cobalamin receptor)	0.89	0.001

* Indicates that gene expression was confirmed by real-time quantitative RT-PCR

Table 4: Summary of previously published literature and current findings showing the effects of loss of parathyroids and PTH (*Hoxa3* null), loss of PTH (*Pth* null), loss of parathyroids (*Gcm2* null) loss of PTHrP (*Pthrp* null), and loss of the amino-terminal effects of PTH and PTHrP (*Pthr1* null) on fetal blood calcium, skeletal development and mineralization, placental calcium transfer, serum PTH and plasma PTHrP as assessed in late gestation

	Ionized calcium	**Skeletal Mineralization**	**Skeletal Scaffold Development**	**Placental Calcium Transfer**	**Serum PTH**	**Plasma PTHrP**
***Hoxa3* null**	Below maternal level (72)	Reduced (29)	Normal (29)	Normal (72)	Undetectable (72)	Normal (72)
***Pth* null**	At maternal level Reduced	Reduced (73) Reduced	Modestly abnormal in C57BL/6 background (i.e. Slightly shortened long bones, shorter metacarpals and metatarsals) (73) Normal in Black Swiss background	Normal	Undetectable (73) Undetectable	Normal
***Pthrp* null**	At maternal level (67)	Normal (29)	Abnormal (i.e. Smaller overall, shortened limbs, rounded skull, shortened and disorganized growth plate, accelerated endochondral bone development) (29)	Reduced (67)	Increased (29)	Low (72)
***Pthr1* null**	Well below maternal level (67)	Reduced (29)	Abnormal (i.e. similar to *Pthrp* null, but more severe) (29)	Increased (67)	Increased (29)	Increased (72)
***Gcm2* null**	At maternal level	Reduced	Normal	Increased	Reduced	Normal

Numbers in parentheses indicate citations
Findings from current study

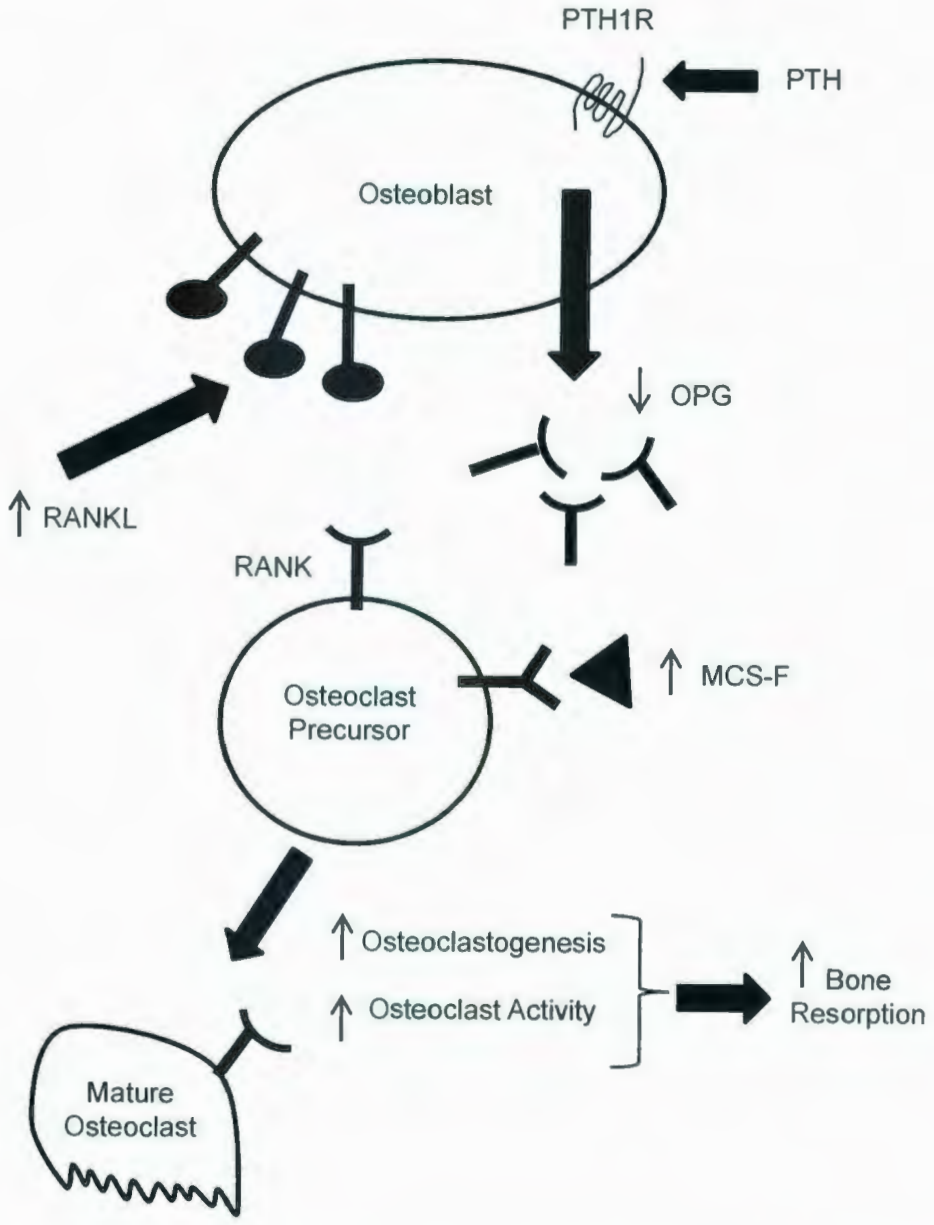


Figure 1: Effect of PTH on the RANK/RANKL/OPG triad in bone. Prolonged or repeated binding of PTH to the PTH1R on osteoblasts, increases the expression of M-CSF and RANKL. As a result, the activity of mature osteoclasts in increased along with the formation of osteoclasts from precursors (osteoclastogenesis). Thus, osteoclastic bone resorption is increased. In addition, PTH decreases the expression of OPG (a soluble decoy receptor that binds to RANKL to prevent it from interacting with RANK), which inhibits osteoclastic bone resorption by reducing osteoclastogenesis and the activity of mature osteoclasts.

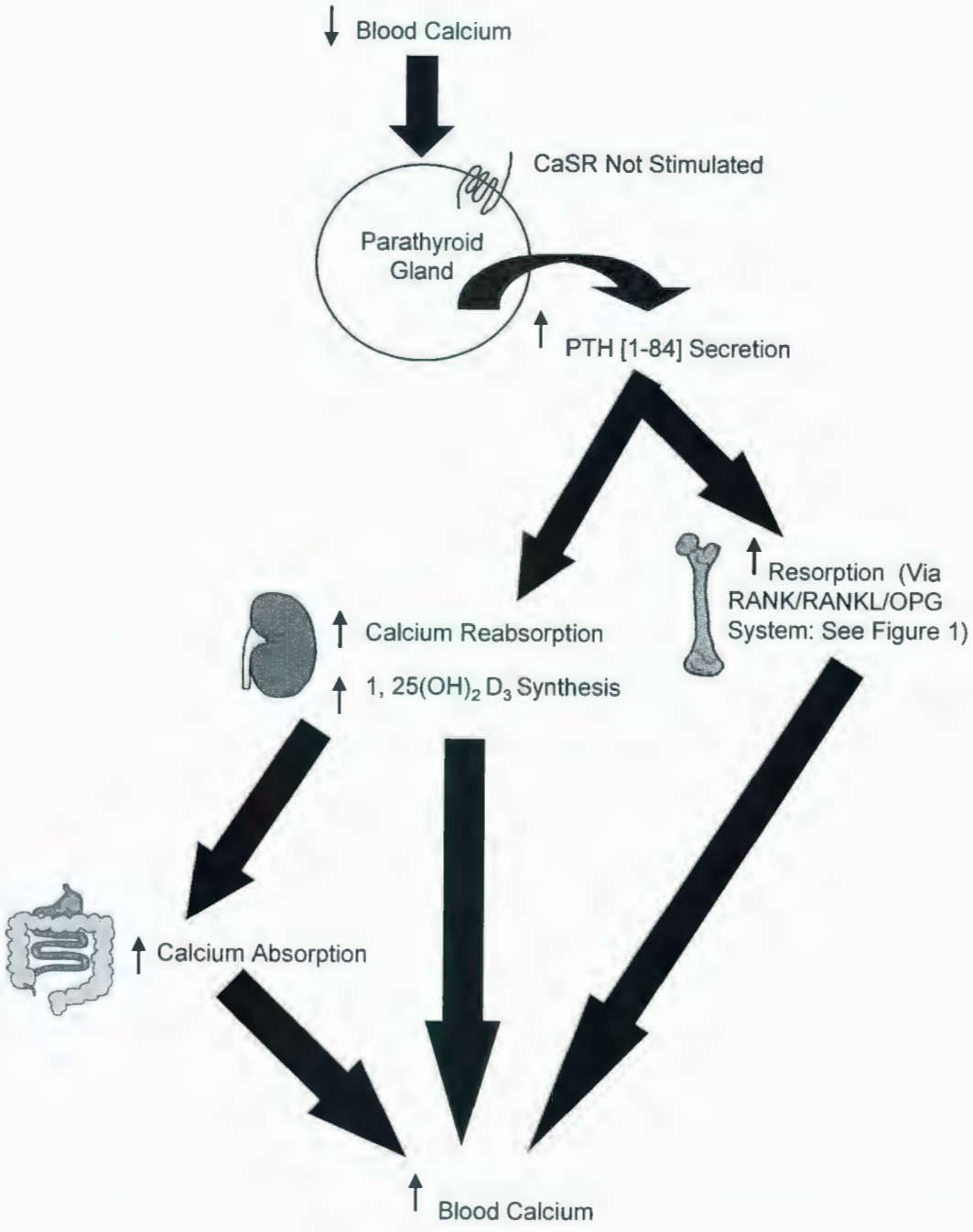


Figure 2: Adult calcium homeostasis. When blood calcium decreases the CaSR on the parathyroid glands is not stimulated, resulting in PTH being released from the parathyroid glands. PTH in the circulation then acts directly on the bone and kidney, and indirectly on the intestine, to return the blood calcium level to normal.

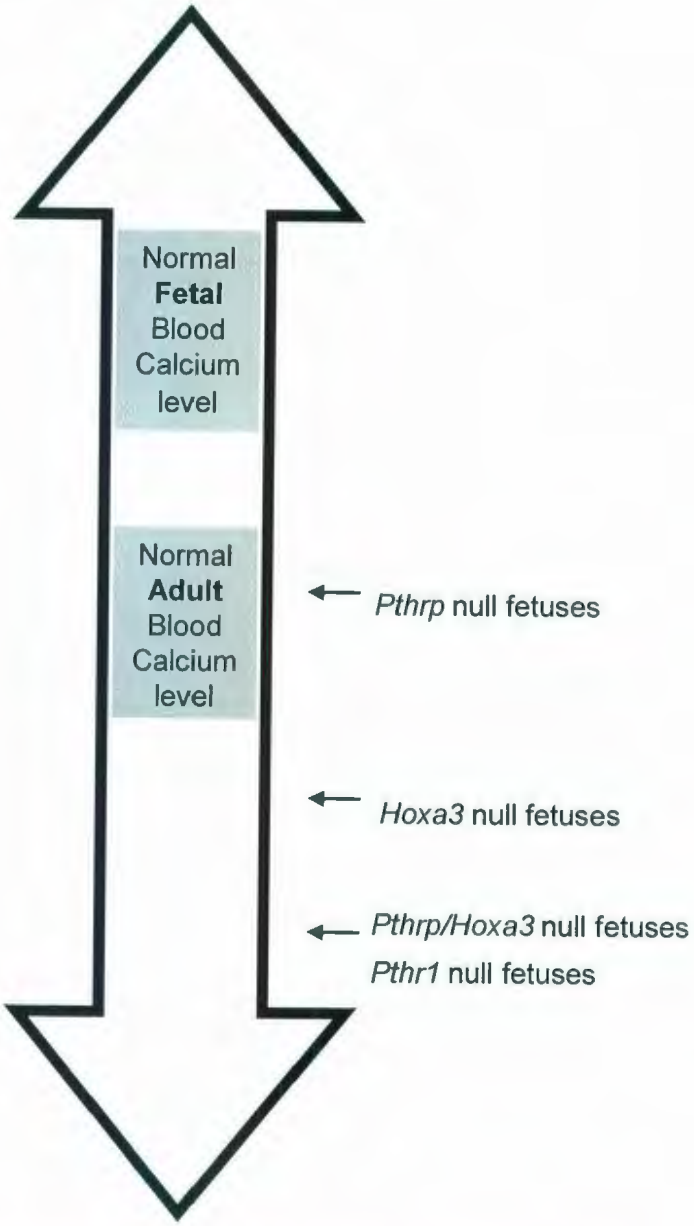


Figure 3: Fetal blood calcium regulation. Blood calcium levels are schematically represented as a continuum on an arrow, with the normal fetal and normal adult levels highlighted in gray. PTH has a more dominant effect on fetal blood calcium regulation than PTHrP as in the absence of PTHrP (*Pthrp* null fetuses), blood calcium declines to the normal adult level (maternal level), while in the absence of PTH and parathyroids (*Hoxa3* null fetuses) the blood calcium declines to well below the maternal level. Interestingly, when both PTH and PTHrP are absent in the fetus (*Pthrp/Hoxa3* null fetuses and *Pthr1* null fetuses), blood calcium declines to a level that is even lower than that observed in the absence of PTH alone.

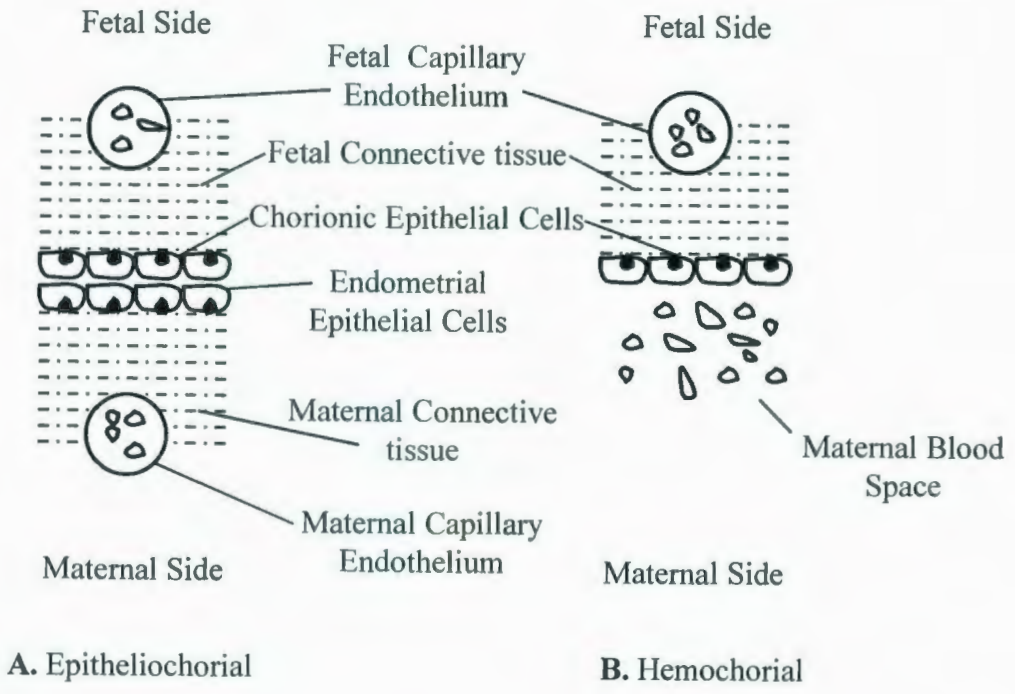


Figure 4: Schematic representation of the tissue layers composing (A) epitheliochorial and (B) hemochorial type placentas. In the epitheliochorial placenta, all 3 fetal tissue layers (capillary endothelium, connective tissue and chorionic epithelial) and all 3 maternal layers of tissue (capillary endothelium, connective tissue and endometrial epithelial) remain. In the hemochorial placenta, all the maternal tissue layers have been eroded and the maternal blood bathes the chorionic epithelial layer.

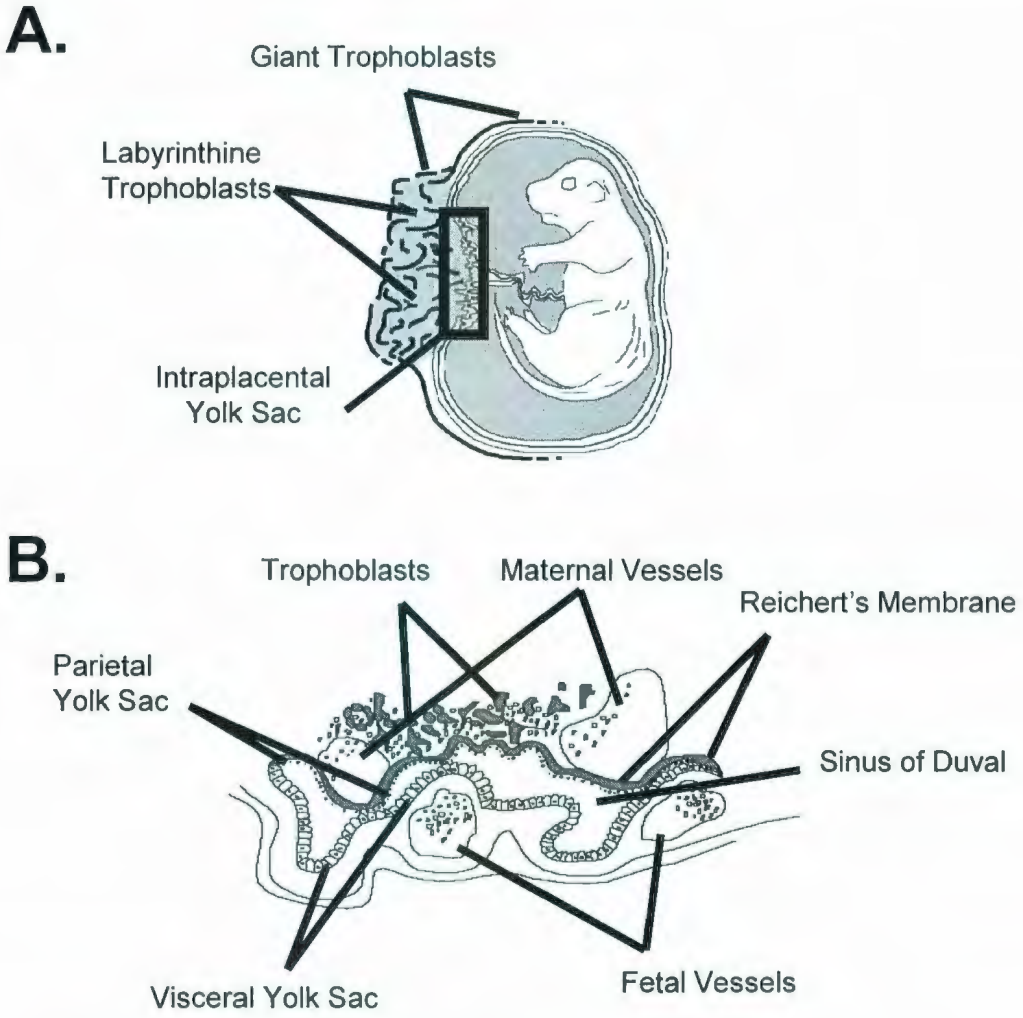


Figure 5: Schematic illustrating the location and anatomy of the intraplacental yolk sac (IPYS). (A) The IPYS is positioned between the maternal and fetal blood spaces in the placenta, such that the parietal layer and Reichert's membrane are in contact with the dome of the placenta, while the visceral yolk sac layer is positioned next to this layer, but separated by the yolk sac cavity. **(B)** In the IPYS, Reichert's membrane and the parietal yolk sac layer (cuboidal cells) overlie the maternal blood spaces and vessels, whereas the visceral yolk sac layer (columnar cells) overlies the fetal blood vessels. The sinus of Duval, which communicates with the yolk sac cavity and the uterine lumen, is located between the two yolk sac layers. (*Reprinted from Kovacs et al. Calcitropic gene expression suggests a role for the intraplacental yolk sac in maternal-fetal calcium exchange. Am J Physiol Endocrinol Metab. 2002 Mar;282(3):E721-32, Copyright (2002), used with permission from The American Physiological Society* (**Appendix A**).

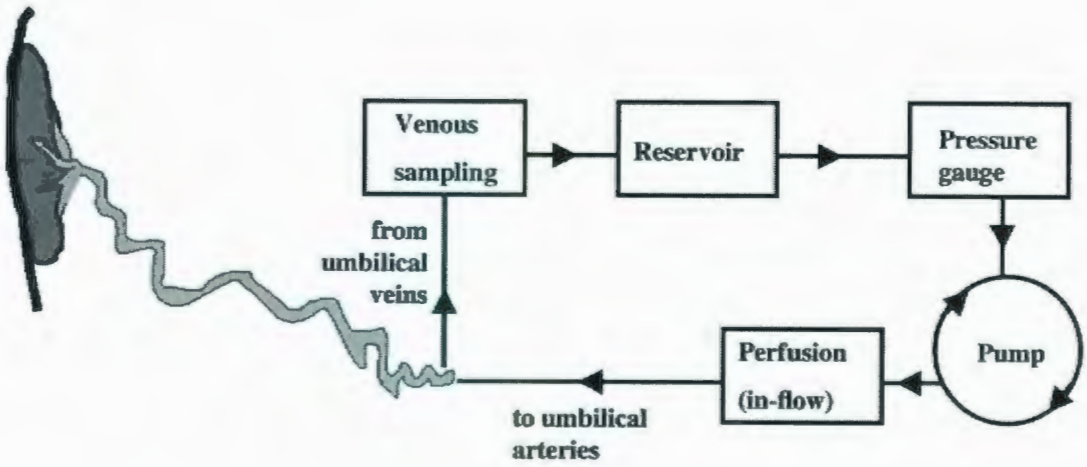


Figure 6: Placental perfusion technique. In this technique, the fetus is completely removed from the uterus. The umbilical vessels are then connected to a semiclosed circuit, where the reservoir contains a blood substitute or autologous fetal blood, and perfusion of the placenta occurs *in situ.* The effect of peptides on maternal-fetal calcium transfer can be tested by administering them to the circuit on the arterial side, and then sampling on the venous side of the circuit (*Reprinted from Pediatric Bone, Kovacs C.S., Chapter 11: Fetal Mineral Homeostasis, pp 271-296, Copyright (2003), with permission from Elsevier* (**Appendix B**).

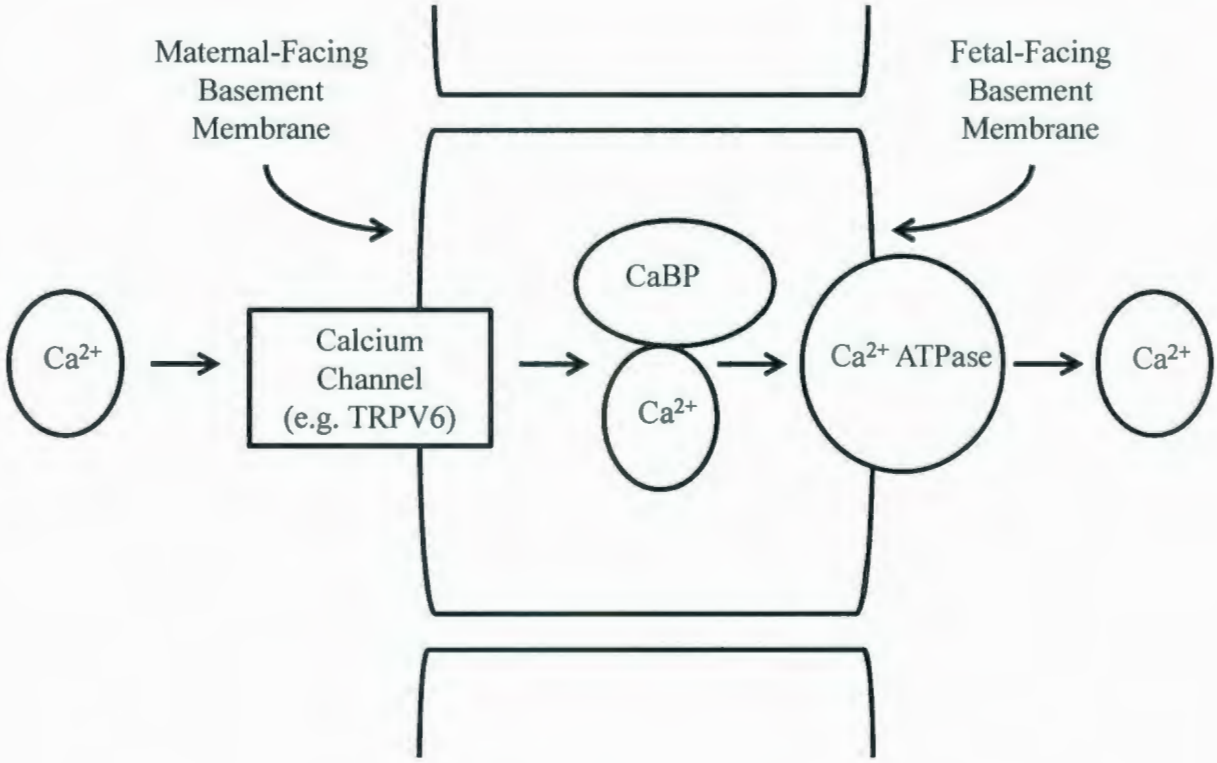


Figure 7: Schematic illustrating the process of active placental calcium transport. In both humans and rodents calcium enters the cell through calcium channels (e.g. TRPV6) on the surface of the maternal-facing basement membrane. Calcium is then shuttled across the cell by calcium binding proteins (e.g. CaBP9k) and finally it is actively extruded from the cell at the fetal-facing basement membrane surface by the active calcium pump (Ca^{2+}-ATPase).

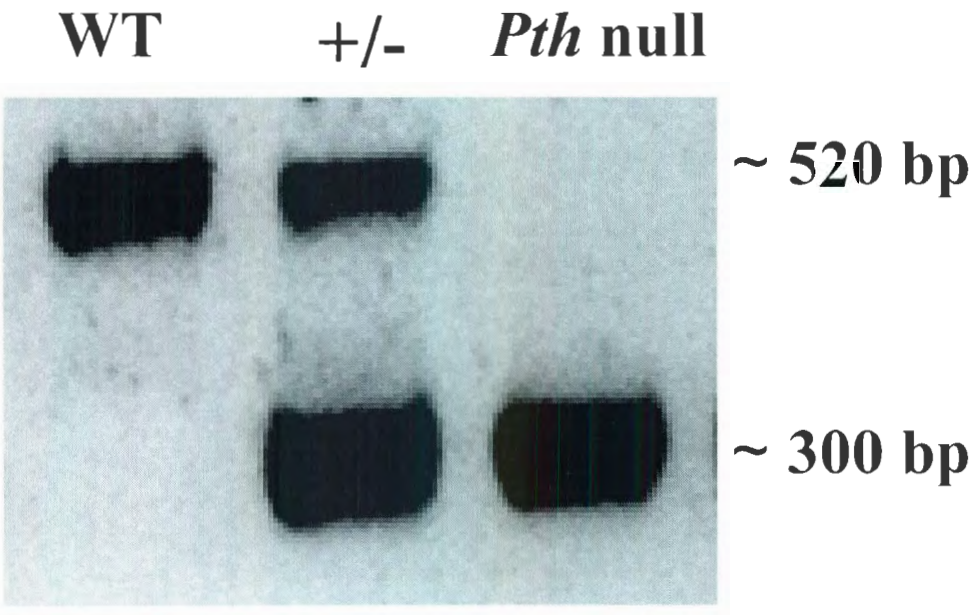


Figure 8: Genotyping of the *Pth* mouse colony by PCR and gel electrophoresis. A single 520 bp band indicated that the mouse was WT (+/+), a single 300 bp band indicated that the mouse was null for the *Pth* gene (-/-), while the presence of both bands indicated that the mouse was HET (+/-) for the *Pth* gene.

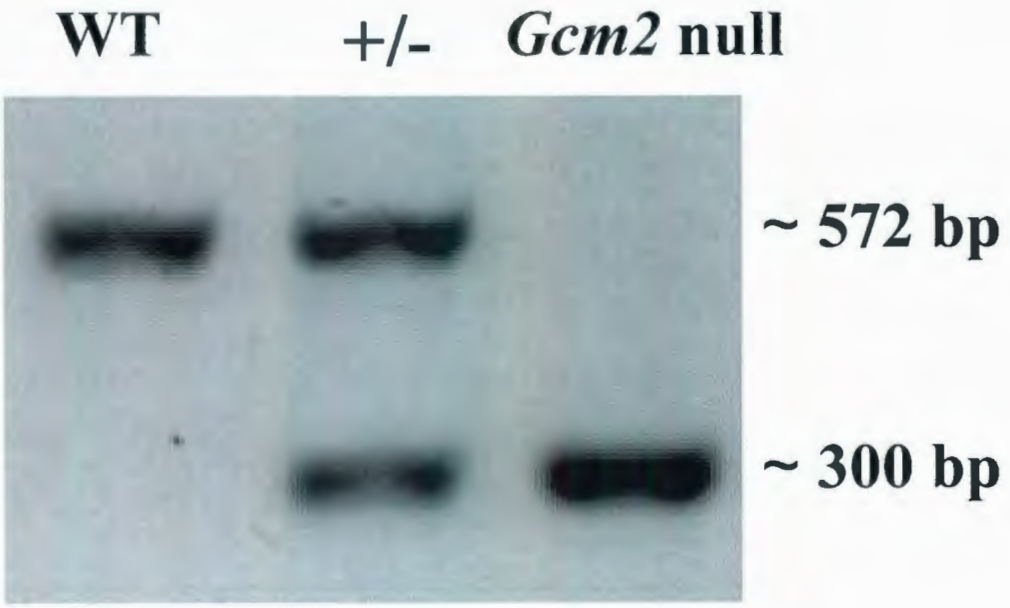


Figure 9: Genotyping of the *Gcm2* mouse colony by PCR and gel electrophoresis. A single 572 bp band indicated that the mouse was WT (+/+), a single 300 bp band indicated that the mouse was null for the *Gcm2* gene (-/-), while the presence of both bands indicated that the mouse was HET (+/-) for the *Gcm2* gene.

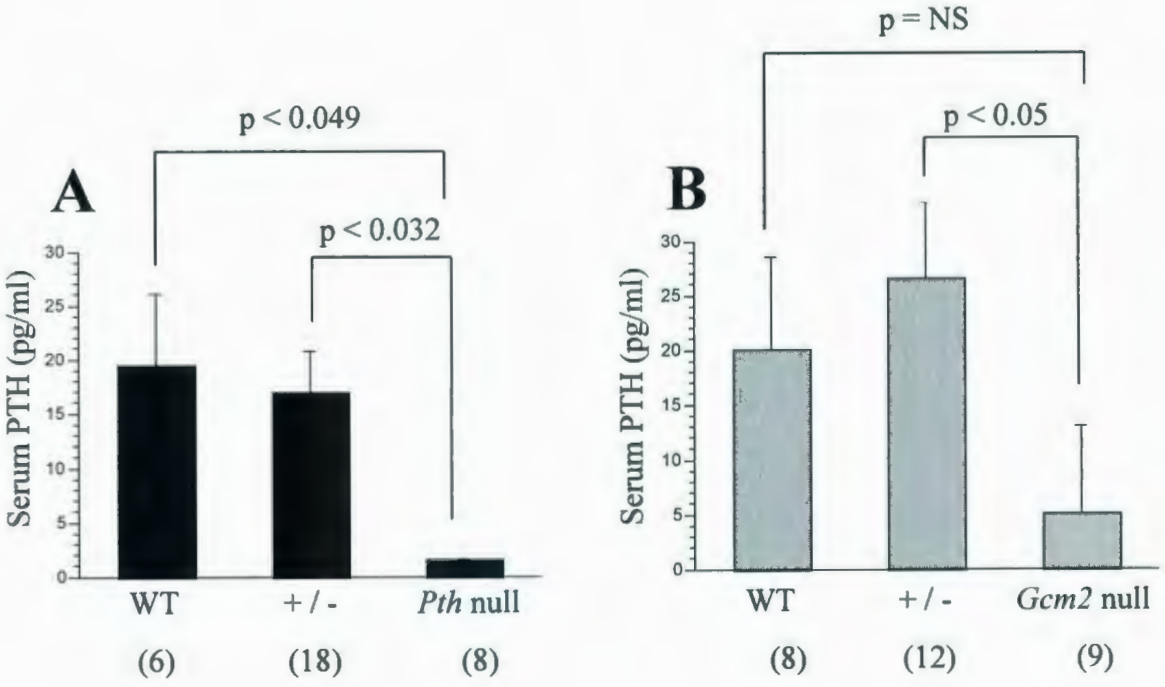


Figure 10: Serum PTH (mean ± SE) in fetuses (ED 18.5) obtained from (A) *Pth* +/- mothers and (B) *Gcm2* +/- mothers. Serum PTH in the *Pth* nulls was lower (reduced to the level of detection) as compared to *Pth* +/- and WT fetuses. Similarly, serum PTH in the *Gcm2* nulls was reduced as compared to *Gcm2* +/- and WT fetuses. The numbers in parentheses indicate the number of fetuses studied.

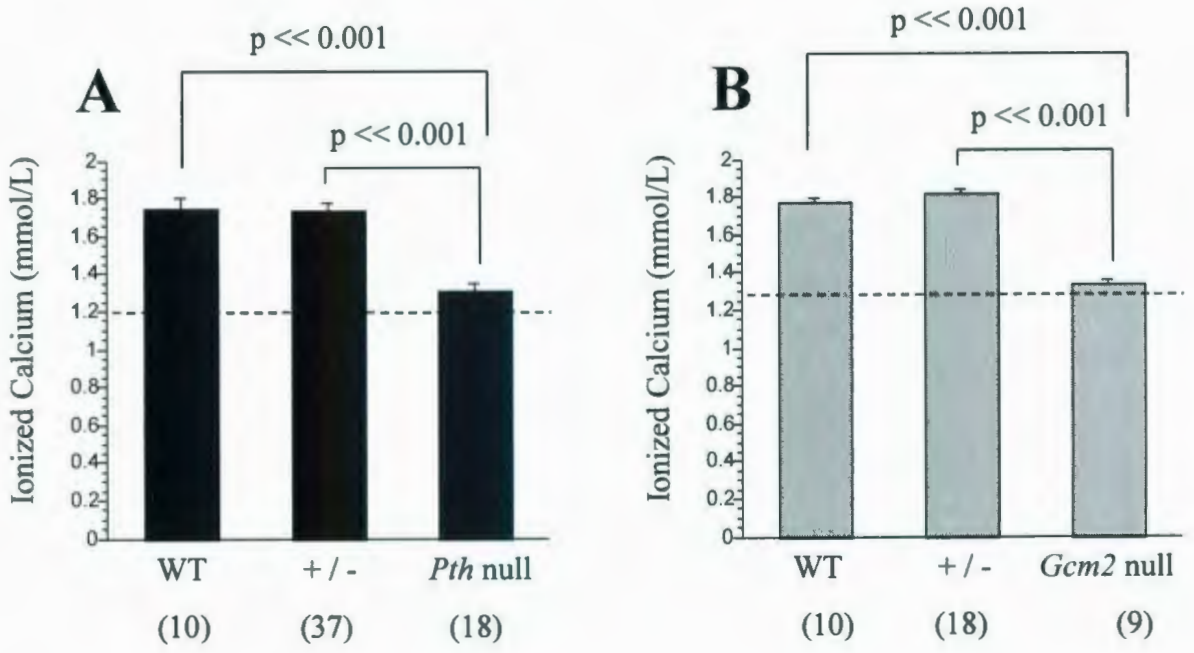


Figure 11: Ionized calcium (mean ± SE) in fetuses (ED 18.5) obtained from (A) *Pth* +/- mothers (B) *Gcm2* +/- mothers. The mean maternal ionized calcium is shown by the dashed line. Both *Pth* null fetuses and *Gcm2* null fetuses had an ionized calcium that was decreased, to approximately the maternal level, as compared to WT and +/- fetuses. The numbers in parentheses indicate the number of fetuses studied.

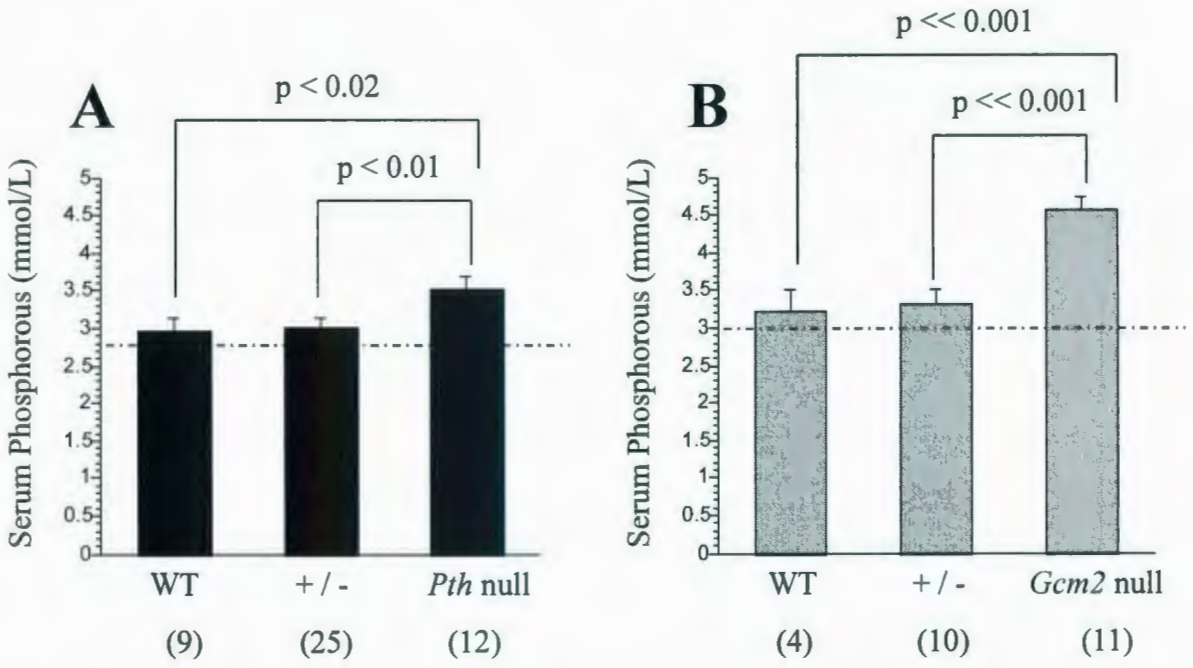


Figure 12: Serum phosphorous (mean ± SE) in fetuses (ED 18.5) obtained from (A) *Pth* +/- mothers (B) *Gcm2* +/- mothers. The mean maternal phosphorous is shown by the dashed line. Serum phosphorus was significantly increased in both *Pth* null fetuses and *Gcm2* null fetuses as compared the respective WT and +/- fetuses. The numbers in parentheses indicate the number of fetuses studied.

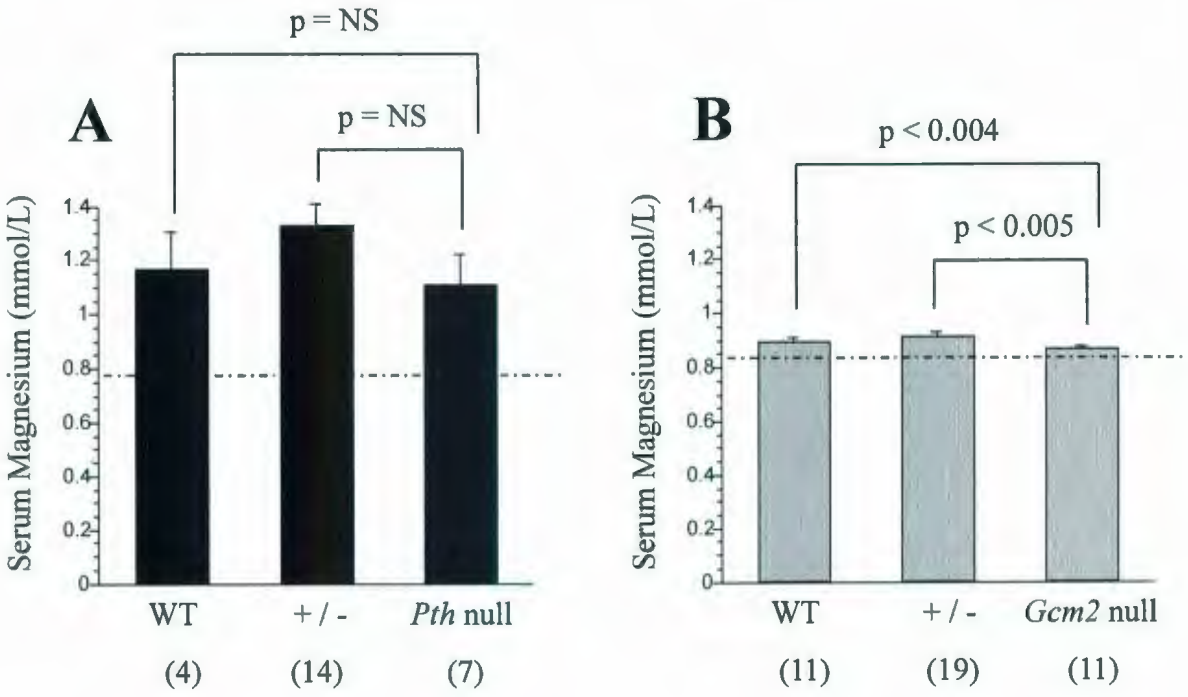


Figure 13: Serum magnesium (mean ± SE) in fetuses (ED 18.5) obtained from (A) *Pth* +/- mothers and (B) *Gcm2* +/- mothers. The mean maternal magnesium is shown by the dashed line. Serum magnesium was slightly reduced in the *Pth* nulls as compared to both WT and *Pth* +/-. In the *Gcm2* nulls, serum magnesium was significantly reduced as compared to WT and *Gcm2* +/- fetuses. The numbers in parentheses indicate the number of fetuses studied.

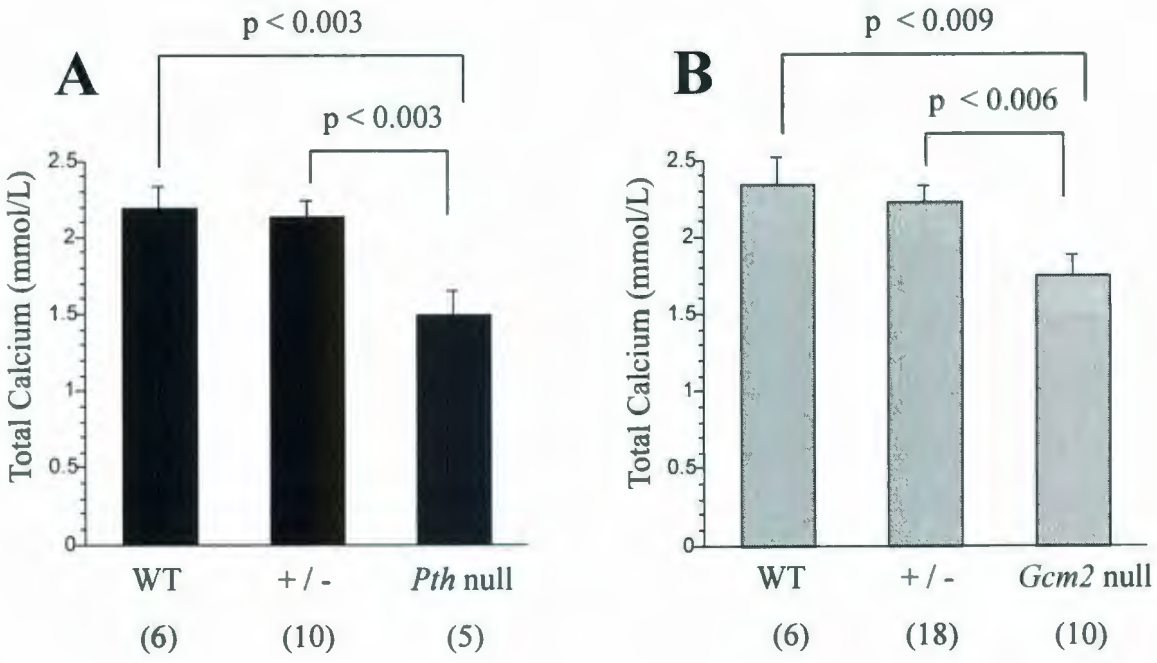


Figure 14: Amniotic fluid total calcium (mean ± SE) in fetuses (ED 18.5 or 17.5) obtained from (A) *Pth* +/- mothers and (B) *Gcm2* +/- mothers. Total calcium in the amniotic fluid was significantly less in both *Pth* null and *Gcm2* null as compared to the respective WT and +/- fetuses. The numbers in parentheses indicate the number of fetuses studied.

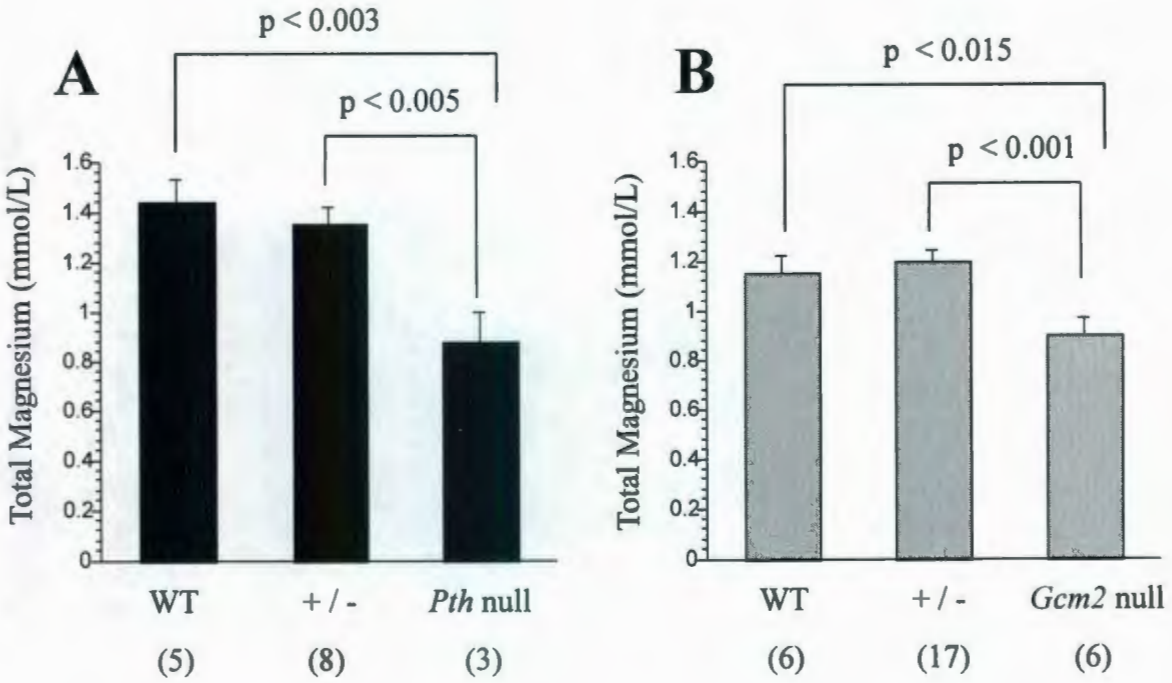
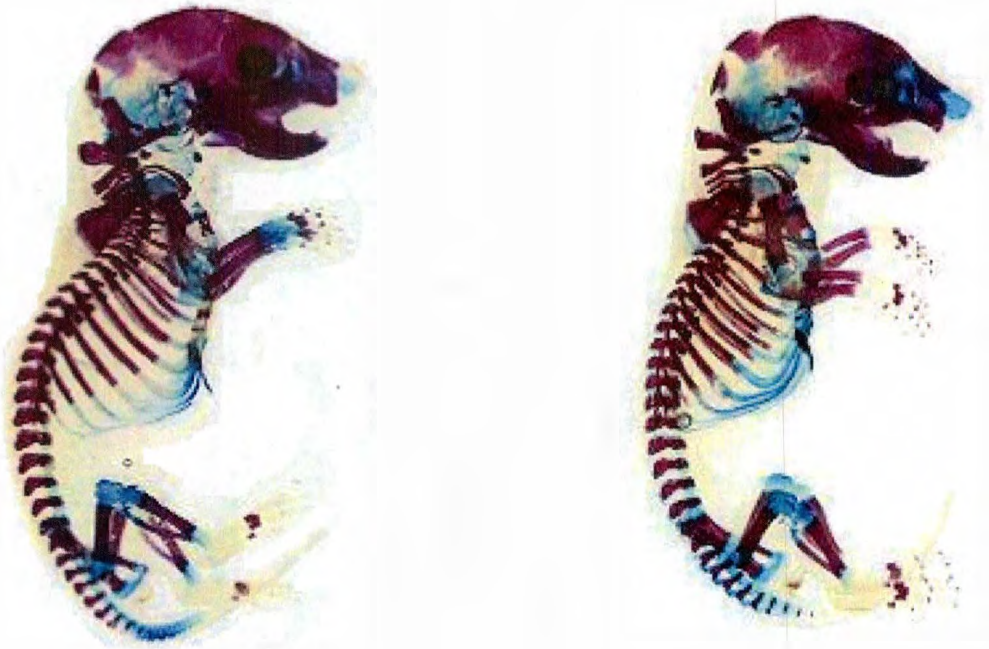


Figure 15: Amniotic fluid total magnesium (mean ± SE) in fetuses (ED 18.5 or 17.5) obtained from (A) *Pth* +/- mothers and (B) *Gcm2* +/- mothers. Total magnesium in the amniotic fluid was significantly less in both *Pth* nulls and *Gcm2* nulls as compared to the respective WT and +/- fetuses. The numbers in parentheses indicate the number of fetuses studied.

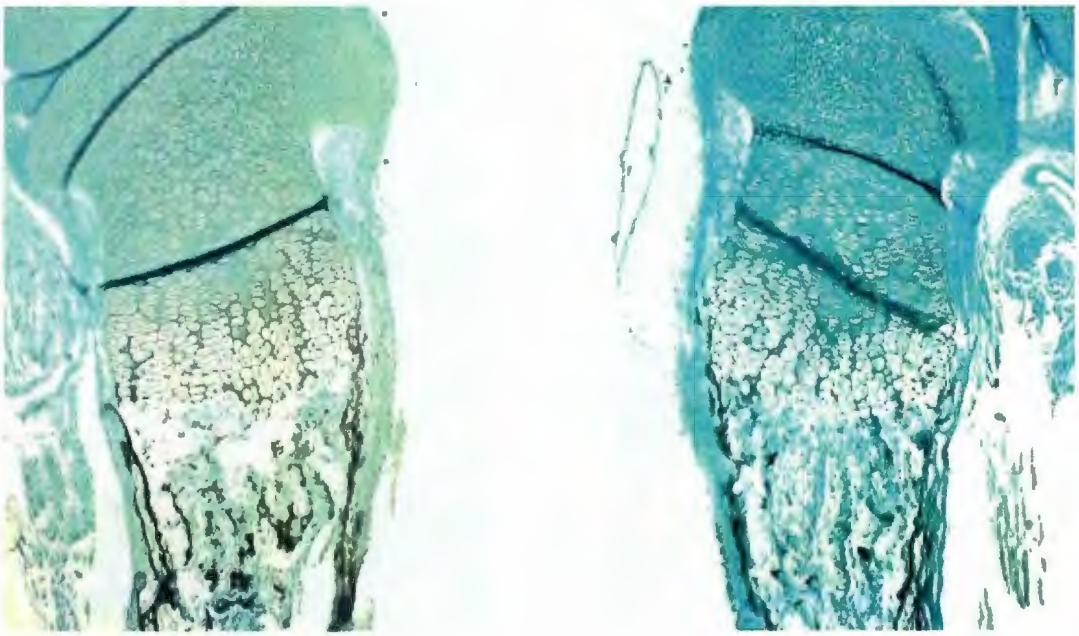


WT *Pth* null

Figure 16: Representative pictures of intact *Pth* null and WT fetal skeletons (ED 18.5) stained with alcian blue (for catilage) and alizarin red (for mineralized tissue). Fetuses obtained from *Pth* +/- mothers. Skeletal morphology, size and mineralization pattern was no different between *Pth* null (n=3) and WT (n=3) fetuses.

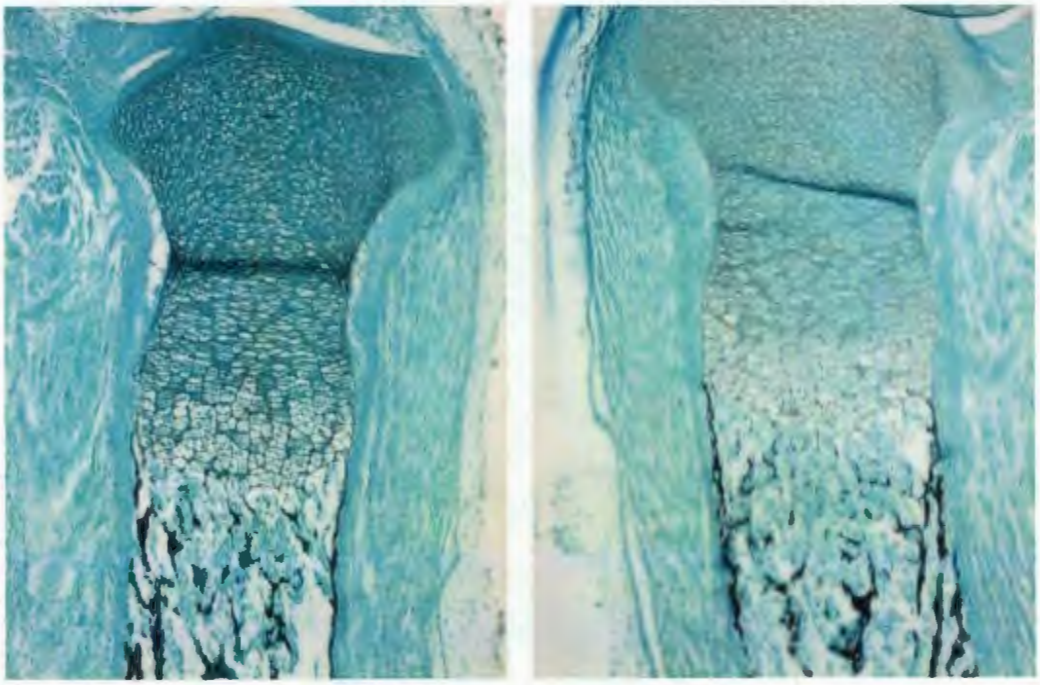
WT *Gcm2* null

Figure 17: Representative pictures of intact *Gcm2* null (n=3) and WT (n=3) fetal skeletons (ED 18.5) stained with alcian blue (for catilage) and alizarin red (for mineralized tissue). Fetuses obtained from *Gcm2* +/- mothers. Skeletal morphology, size and mineralization pattern was no different between *Gcm2* null (n=3) and WT (n=3) fetuses.



WT *Pth* null

Figure 18: Representative sections showing Von Kossa staining of tibias and growth plates that have been counter-stained with methyl green. Fetuses (ED 18.5) obtained from *Pth* +/- mothers. *Pth* null fetuses (n=6) appeared to have a normal amount of mineral deposition (represented by black deposits) within the tibia, and have normal growth plates as compared to WT (n=6) siblings.



WT *Gcm2* null

Figure 19: Representative sections showing Von Kossa staining of tibias and growth plates that have been counter-stained with methyl green. Fetuses (ED 18.5) obtained from *Gcm2* +/- mothers. *Gcm2* null fetuses (n=6) appeared to have a normal amount of mineral deposition (represented by black deposits) within the tibia, and have normal growth plates as compared to WT (n=6) siblings.

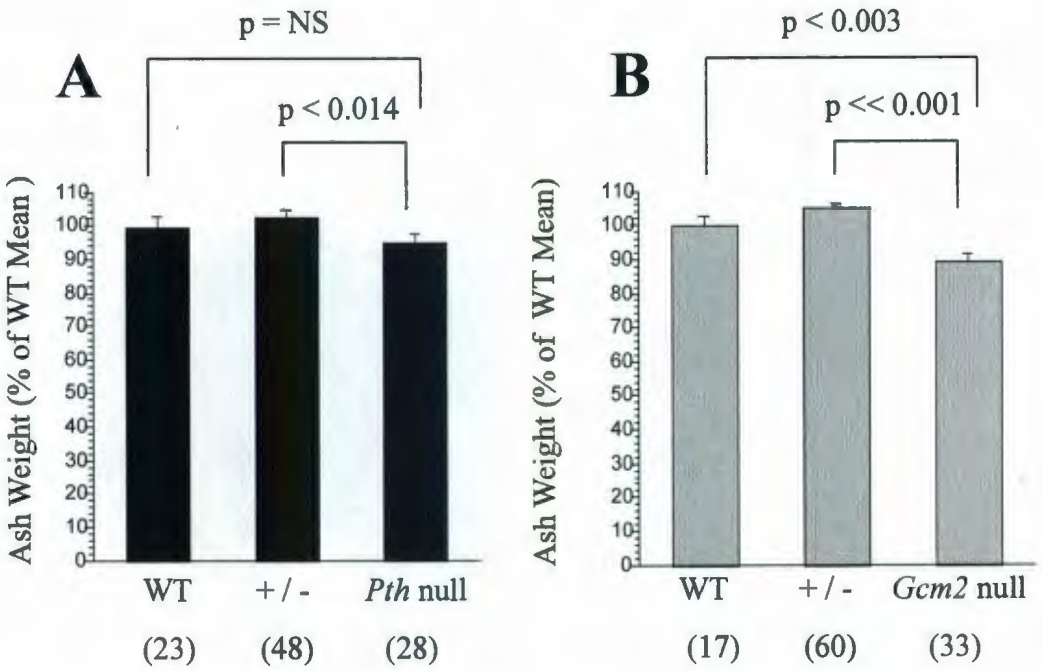


Figure 20: Ash weight, normalized to the mean weight of WT fetuses within each litter (% WT mean ± SE) in fetuses (ED 18.5) obtained from (A) *Pth* +/- mothers and (B) *Gcm2* +/- mothers. Ash weight was reduced in both *Pth* nulls and *Gcm2* nulls. In the *Gcm2* nulls it was significantly reduced as compared to both WT and +/- fetuses, while *Pth* nulls only differed significantly from *Pth* +/- fetuses. The numbers in parentheses indicate the number of fetuses studied.

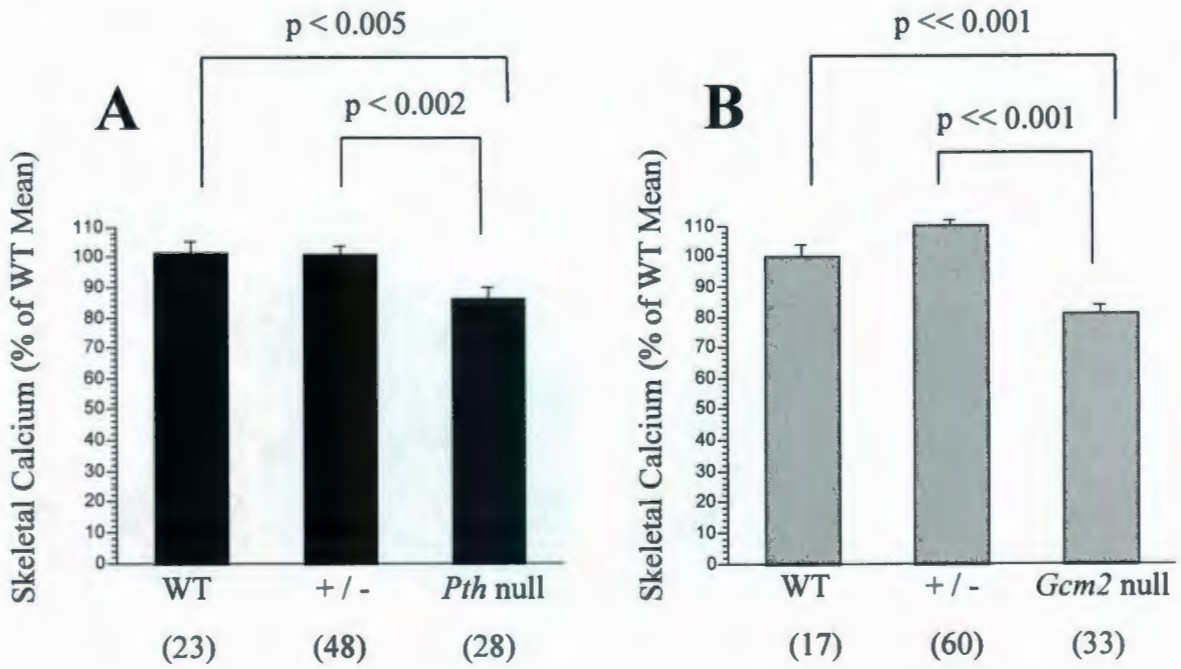


Figure 21: Skeletal calcium content, normalized to the mean skeletal calcium content of WT fetuses within each litter (% WT mean ± SE) in fetuses (ED 18.5) obtained from (A) *Pth* +/- mothers and (B) *Gcm2* +/- mothers. Skeletal calcium was significantly reduced in both *Pth* and *Gcm2* nulls, as compared to the respective WT and +/- fetuses. The numbers in parentheses indicate the number of fetuses studied.

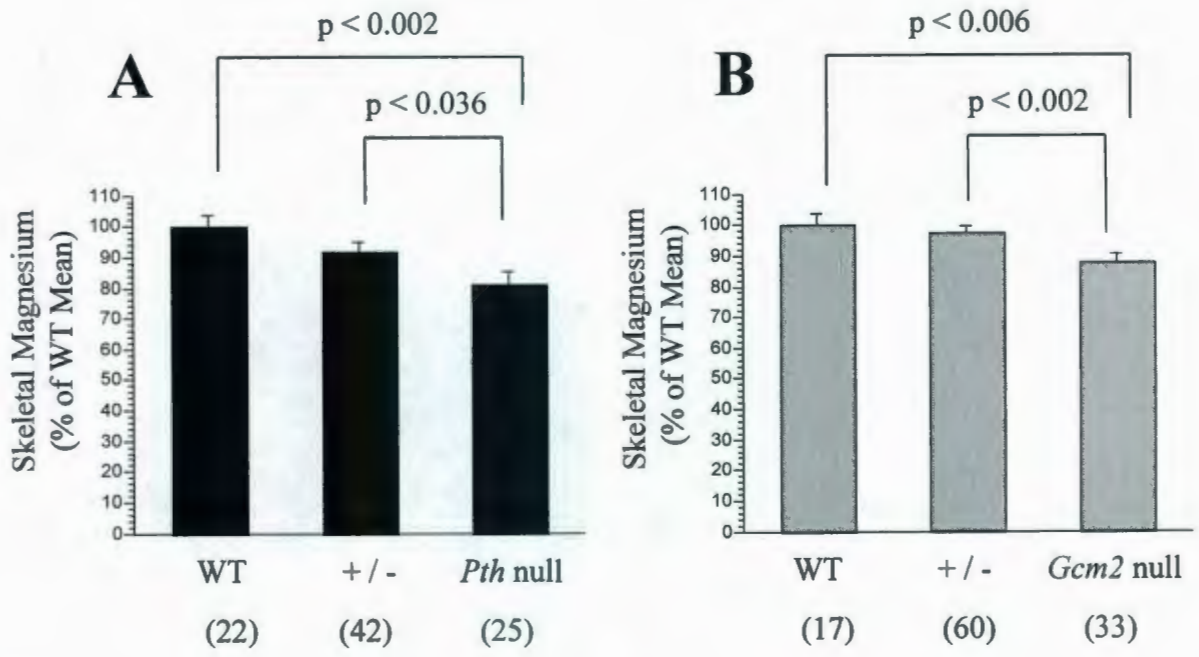


Figure 22: Skeletal magnesium content, normalized to the mean skeletal magnesium content of WT fetuses within each litter (% WT mean ± SE) in fetuses (ED 18.5) obtained from (A) *Pth* +/- mothers and (B) *Gcm2* +/- mothers. Skeletal magnesium was significantly reduced in both *Pth* and *Gcm2* nulls, as compared to the respective WT and +/- fetuses. The numbers in parentheses indicate the number of fetuses studied.

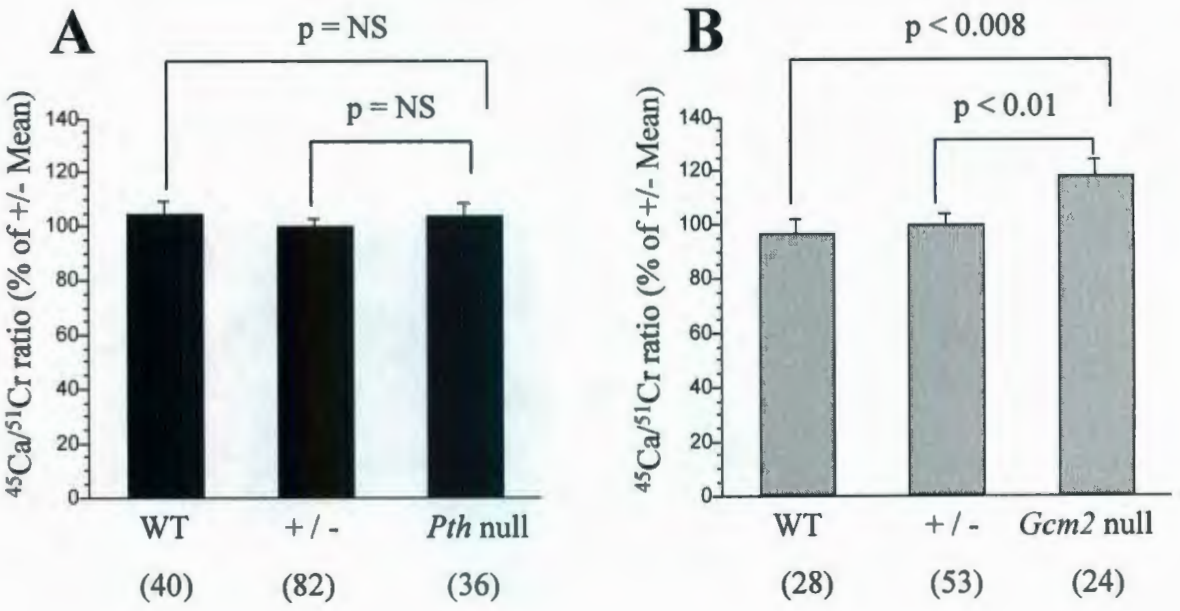


Figure 23: $^{45}Ca/^{51}Cr$ activity accumulated in each fetus at 5 minutes, normalized to the mean value of the heterozygotes within each litter (% HET mean ± SE). Fetuses (ED 17.5 or 18.5) obtained from (A) *Pth* +/- mothers and (B) *Gcm2* +/- mothers. Placental calcium transfer was no different in *Pth* null fetuses as compared to WT and *Pth* +/- fetuses. In contrast, calcium transfer was significantly increased in *Gcm2* null fetuses as compared to WT and *Gcm2* +/- fetuses. The numbers in parentheses indicate the number of fetuses studied.

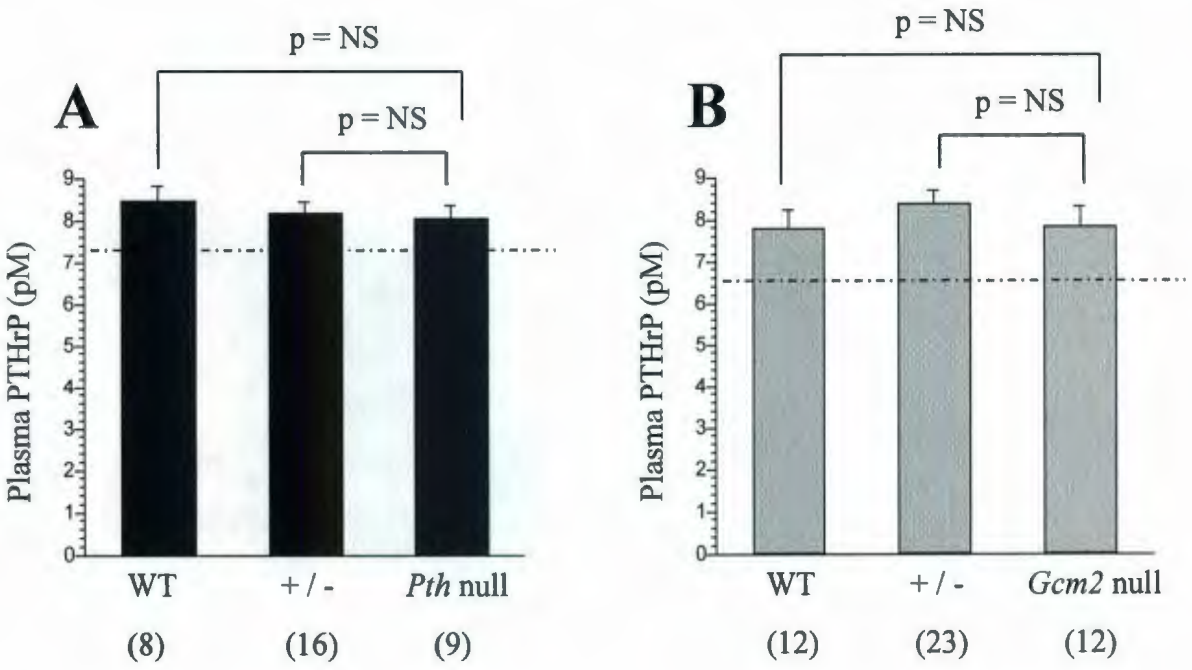


Figure 24: Plasma PTHrP levels (mean ± SE) in fetuses (ED 18.5) obtained from (A) *Pth* +/- mothers and (B) *Gcm2* +/- mothers. The mean maternal PTHrP level is shown by the dashed line. Plasma PTHrP in the *Pth* null and the *Gcm2* null was no different as compared to WT and +/- fetuses. The numbers in parentheses indicate the number of fetuses studied.

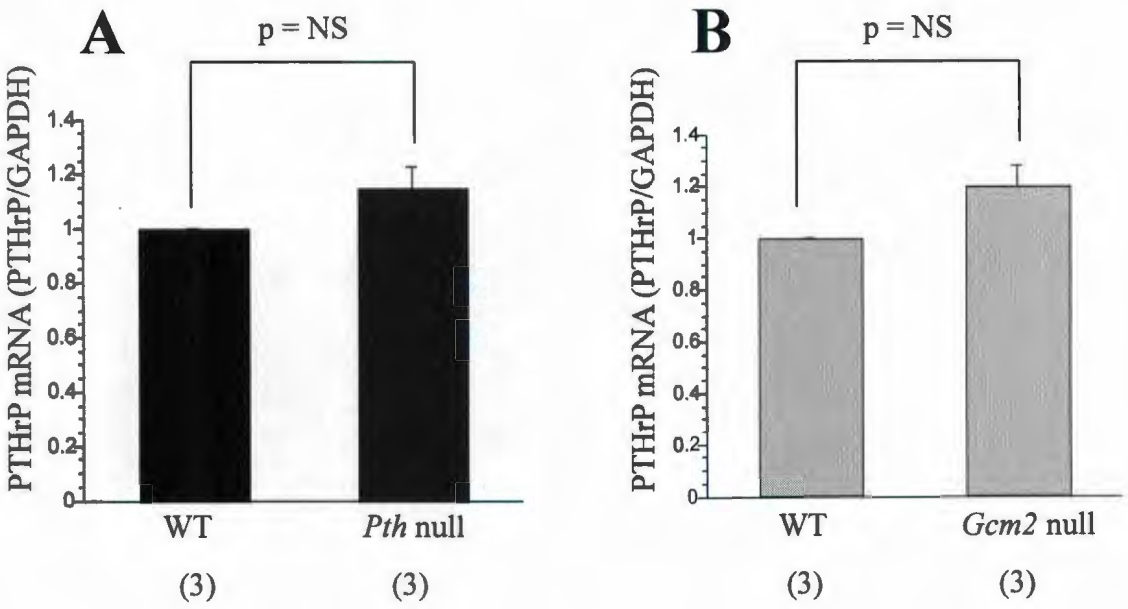


Figure 25: PTHrP mRNA expression (mean fold ± SE) in neck tissue of fetuses (ED 18.5) obtained from (A) *Pth* +/- mothers and (B) *Gcm2* +/- mothers. Real-time quantitative RT-PCR showed that PTHrP expression in the neck of both *Pth* null and *Gcm2* null fetuses was no different as compared to WT fetuses. Tests were performed in triplicate and normalized to a GAPDH control. The numbers in parentheses indicate the number of fetuses studied.

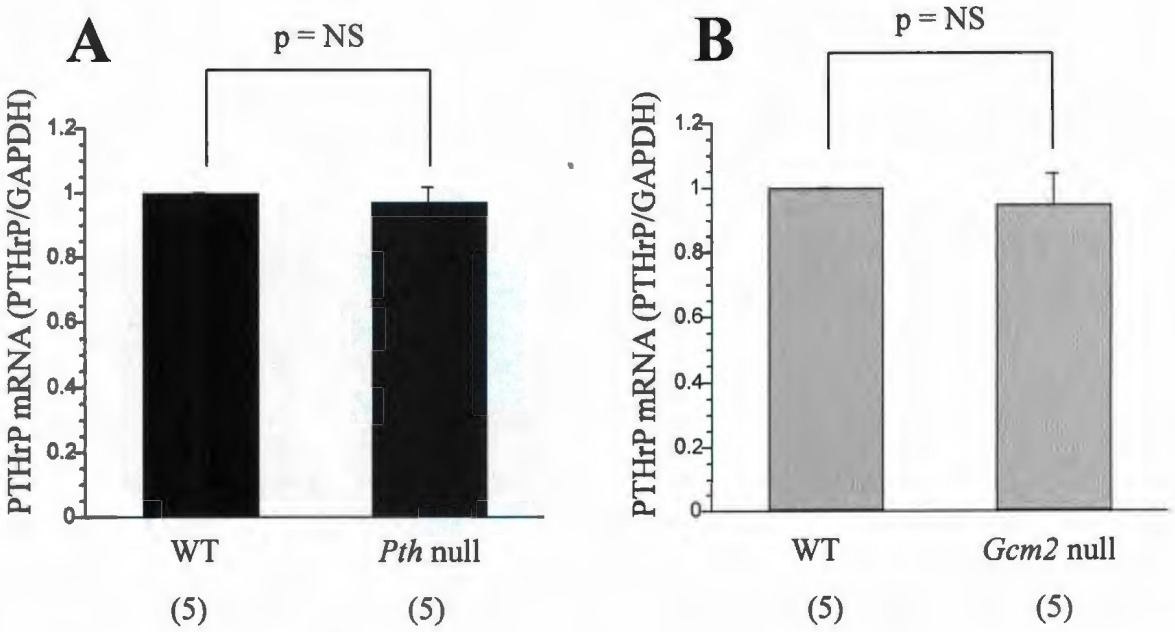


Figure 26: PTHrP mRNA expression (mean fold ± SE) in the placenta of fetuses (ED 18.5) obtained from (A) *Pth* +/- mothers and (B) *Gcm2* +/- mothers. Real-time quantitative RT-PCR showed that PTHrP expression in the placenta of *Pth* null and *Gcm2* null fetuses was no different as compared to WT. Tests were performed in triplicate and normalized to a GAPDH control. The numbers in parentheses indicate the number of fetuses studied.

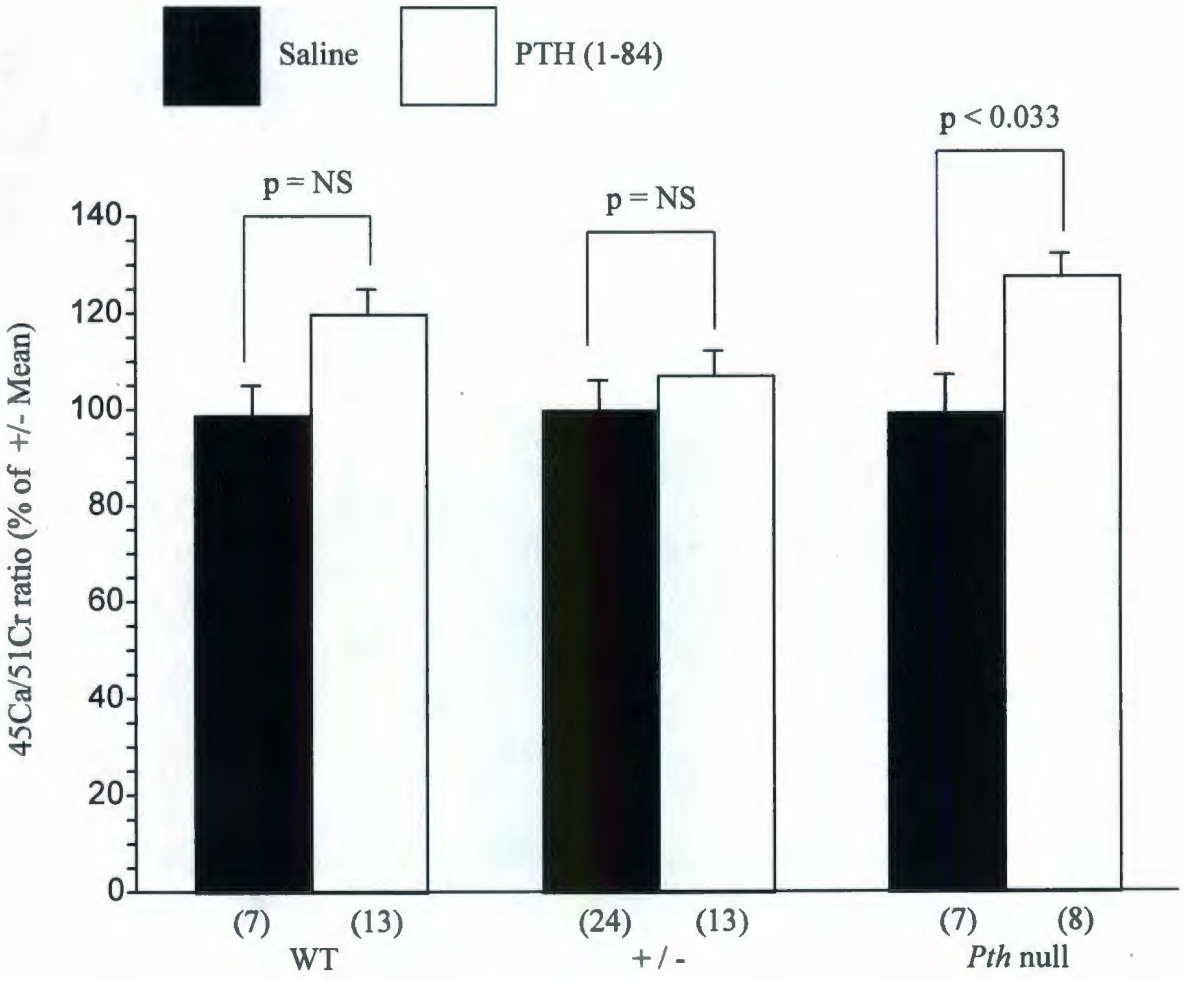


Figure 27: Effect of PTH treatment on placental calcium transfer (% HET mean ± SE). Fetuses (ED 17.5 or 18.5) obtained from *Pth* +/- mothers were treated *in utero* with saline or PTH 1-84. 85 minutes later the isotope was administered and 5 min after that the 45Ca/51Cr ratio was measured. *Pth* nulls increased placental calcium transfer in response to injections of PTH (1-84) as compared to saline. The numbers in parentheses indicate the number of fetuses studied.

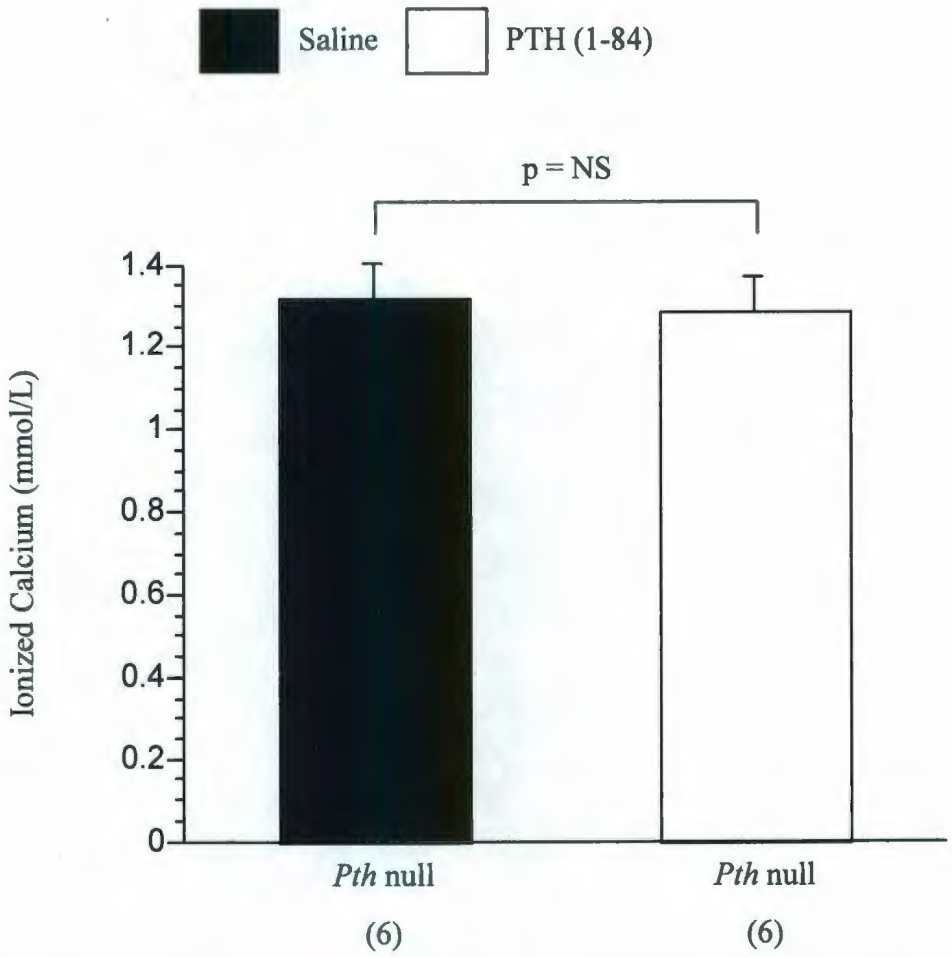


Figure 28: Ionized calcium (mean ± SE) in fetuses (ED 18.5) 90 min after an *in utero* injection of either saline or PTH (1-84). Ionized calcium was no different between *Pth* null fetuses that were treated *in utero* with either saline or PTH (1-84). The numbers in parentheses indicate the number of fetuses studied.

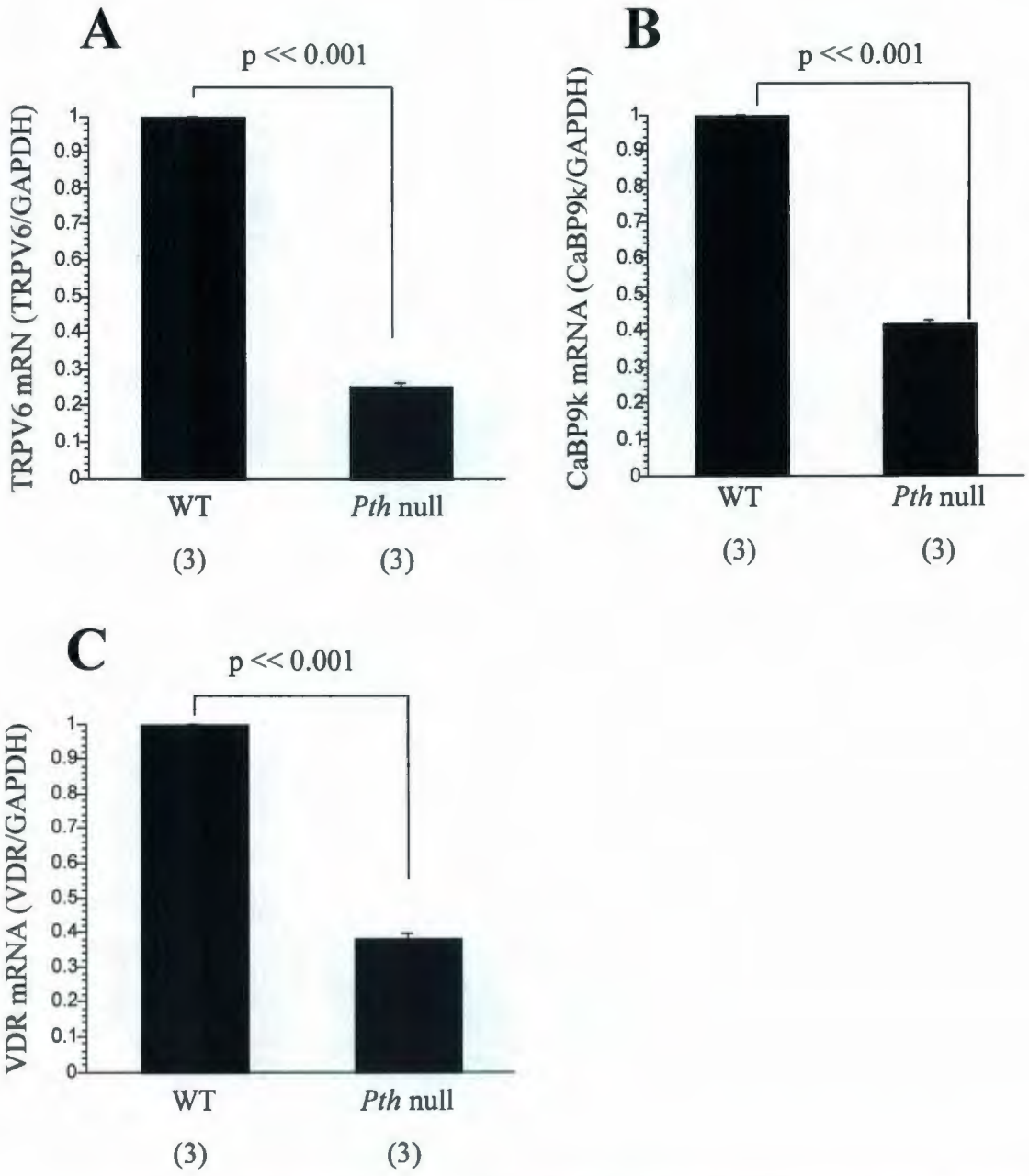


Figure 29: (A) TRPV6 (B) CaBP9k (C) VDR mRNA expression (mean fold ± SE) in placenta harvested from WT and *Pth* null fetuses (ED 18.5) that were treated *in utero* with saline. Real-time quantitative RT-PCR showed that TRPV6, CaBP-9k, and VDR expression in the placenta of saline injected *Pth* null fetuses was significantly decreased as compared to placenta of WT fetuses that were also injected *in utero* with saline. Tests were performed in triplicate and normalized to a GAPDH control. The numbers in parentheses indicate the number of fetuses studied.

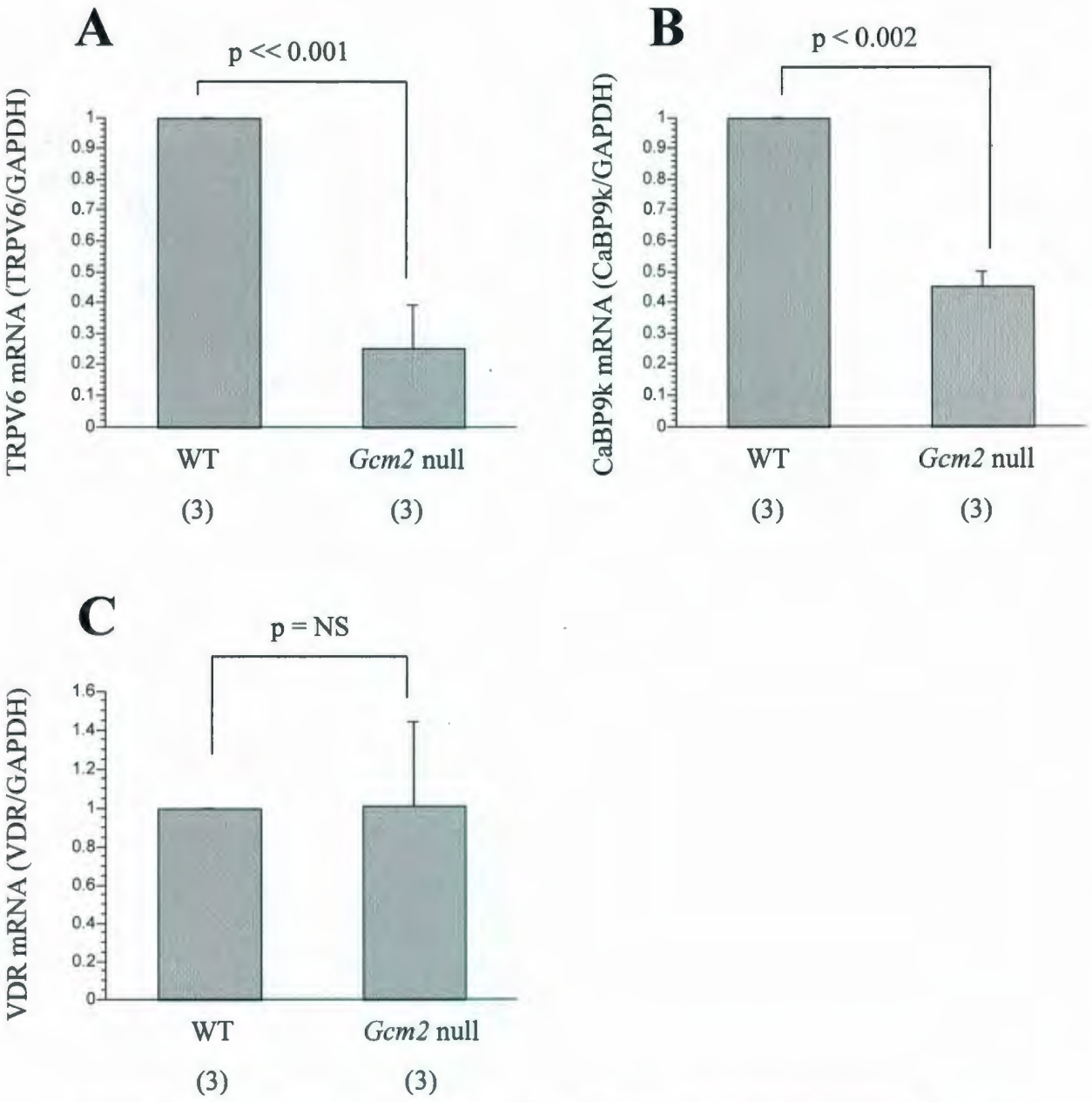


Figure 30: (A) TRPV6 (B) CaBP9k (C) VDR mRNA expression (mean fold ± SE) in placenta harvested from WT and *Gcm2* null fetuses (ED 18.5). Real-time quantitative RT-PCR showed that TRPV6 and CaBP9k expression in the placenta of *Gcm2* null fetuses was significantly decreased as compared to placenta of WT fetuses. VDR expression however was no different between WT and *Gcm2* null placentas. Tests were performed in triplicate and normalized to a GAPDH control. The numbers in parentheses indicate the number of fetuses studied.

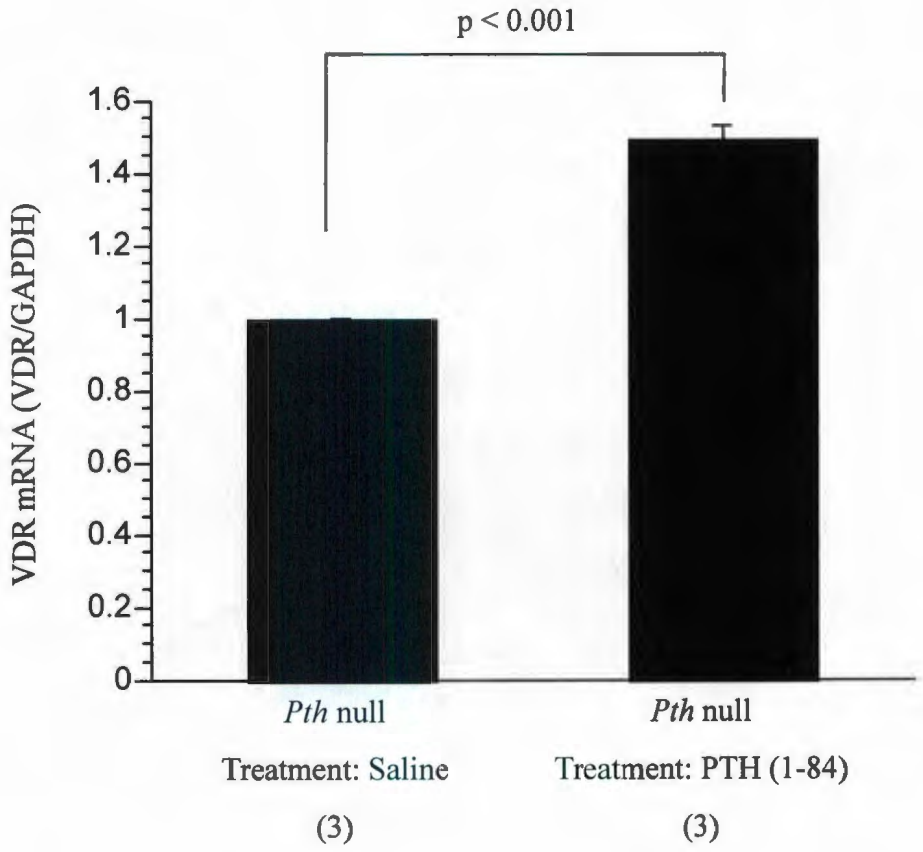


Figure 31: VDR mRNA expression (mean fold ± SE) in placenta harvested from *Pth* null fetuses (ED 18.5) that were treated *in utero* with either PTH 1-84 or saline. Real-time quantitative RT-PCR showed that VDR expression in the placenta of *Pth* null fetuses injected with PTH 1-84 was significantly increased as compared to placenta of *Pth* null fetuses that were injected *in utero* with saline. Tests were performed in triplicate and normalized to a GAPDH control. The numbers in parentheses indicate the number of fetuses studied.

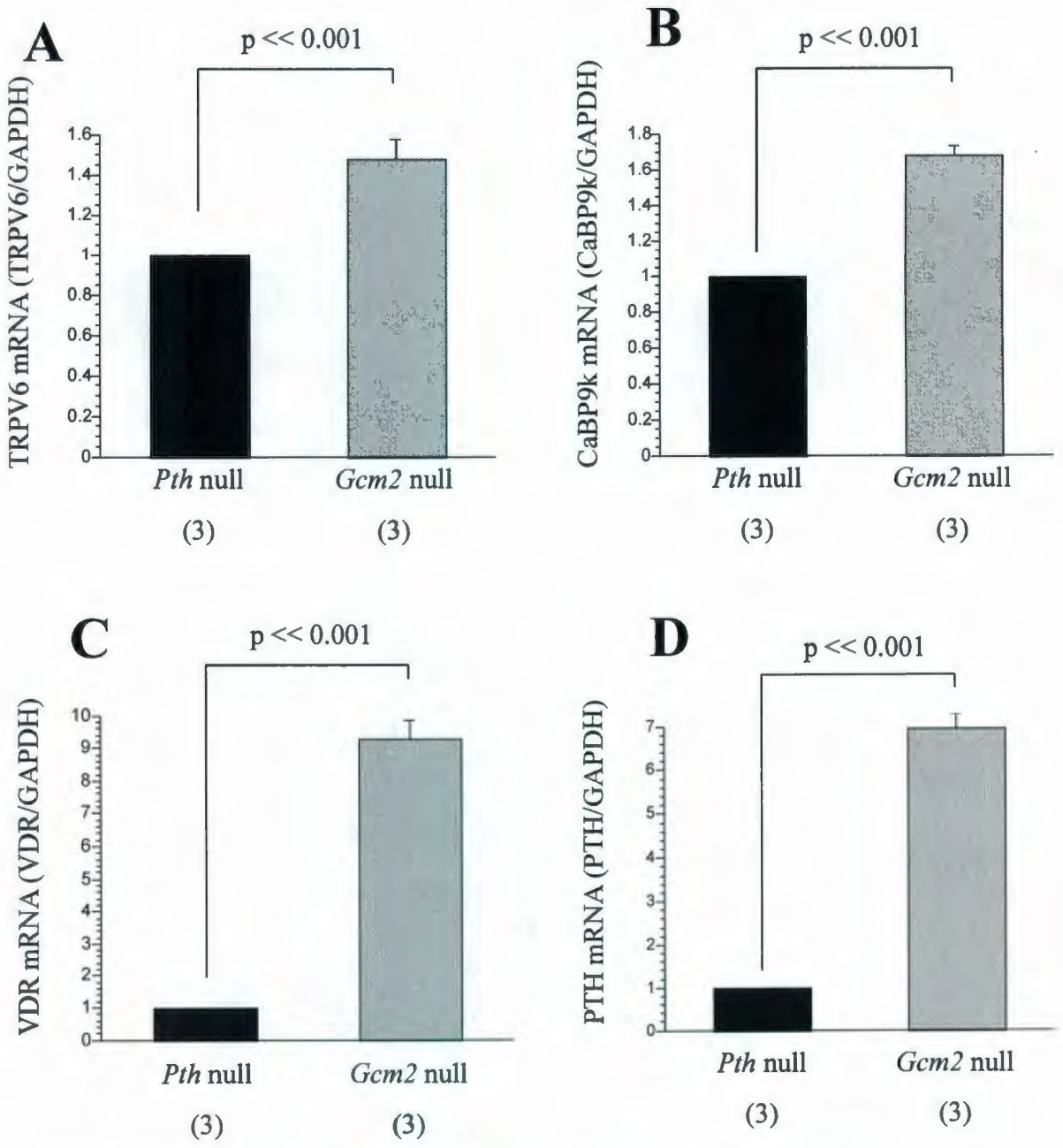


Figure 32: (A)TRPV6 (B) CaBP9k mRNA expression and (C) VDR and (D) PTH mRNA expression (mean fold ± SE) in placenta harvested from *Gcm2* null fetuses (gray bars) as compared to *Pth* null placenta (black bars). Real-time quantitative RT-PCR showed that TRPV6, CaBP9k, VDR, and PTH expression in the placenta of *Gcm2* null fetuses was significantly higher as compared to placenta of *Pth* null fetuses.. Tests were performed in triplicate and normalized to a GAPDH control. The numbers in parentheses indicate the number of fetuses studied.

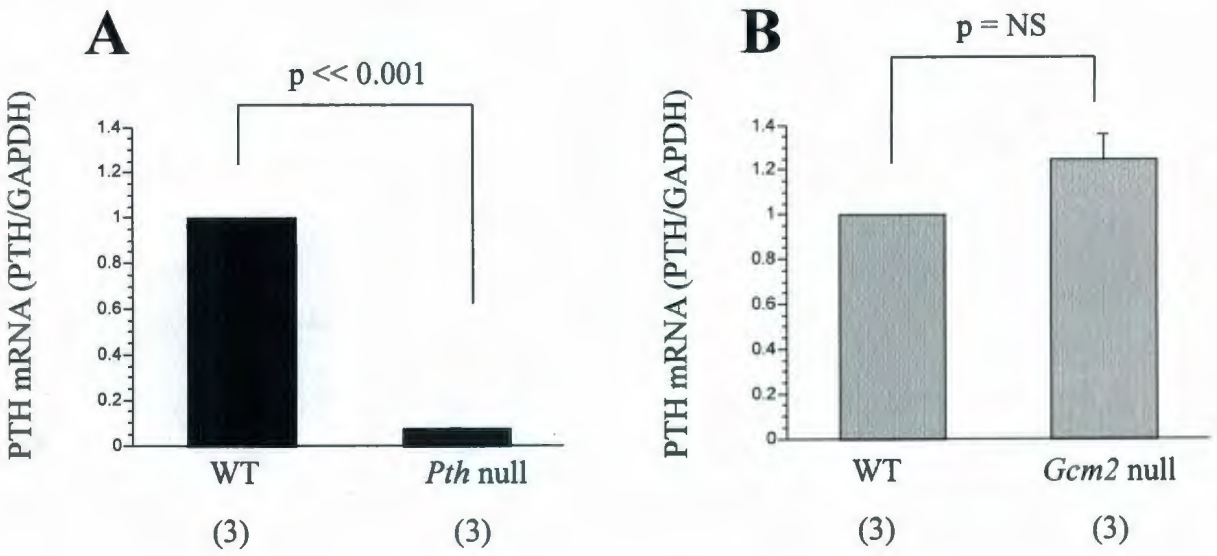


Figure 33: PTH mRNA expression (mean fold ± SE) in the placenta of fetuses (ED 18.5) obtained from (A) *Pth* +/- mothers and (B) *Gcm2* +/- mothers. Real-time quantitative RT-PCR showed that as compared to WT, PTH expression in the placenta of *Pth* nulls was significantly lower. In *Gcm2* null placenta however, PTH expression was slightly (but not significantly) increased as compared to PTH expression in placenta harvested from WT fetuses. Tests were performed in triplicate and normalized to a GAPDH control. The numbers in parentheses indicate the number of fetuses studied.

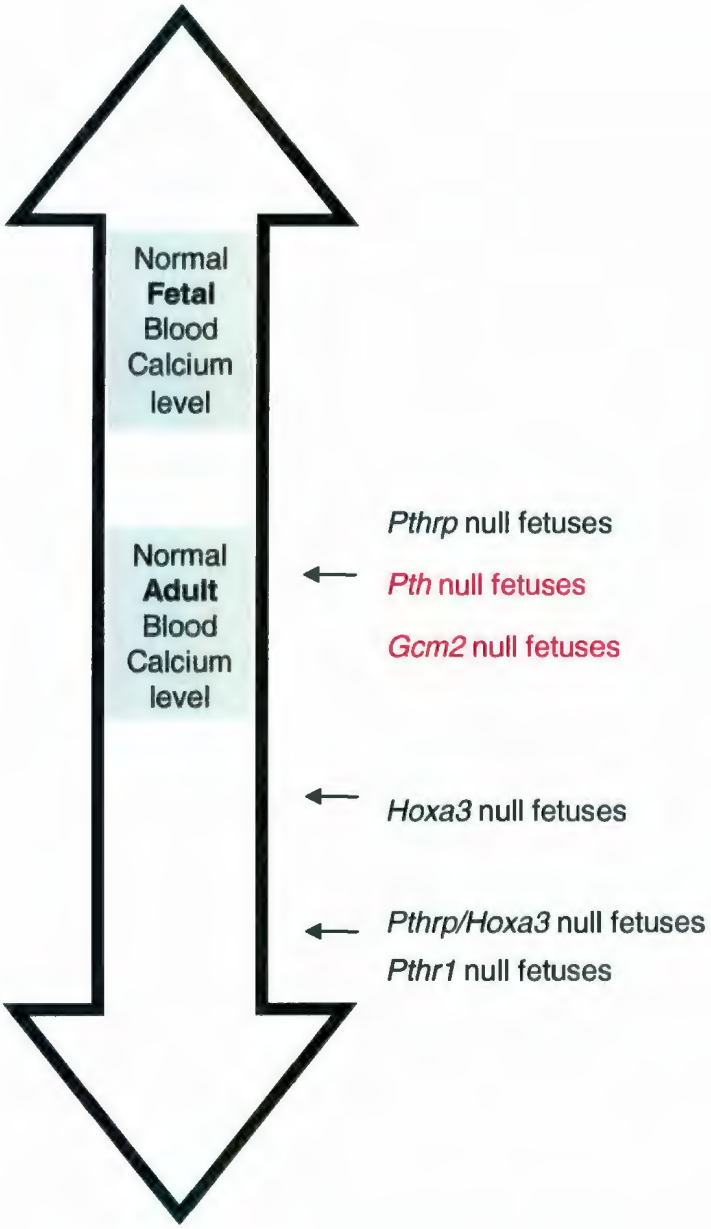


Figure 34: New findings in fetal blood calcium regulation. Blood calcium levels are schematically represented as a continuum on an arrow, with the normal fetal and normal adult levels highlighted in gray. In contrast to what was originally thought, PTH does not have a more dominant effect on fetal blood calcium regulation than PTHrP. In the absence of PTHrP (*Pthrp* null fetuses), absence of PTH (*Pth* null), and reduction of PTH (*Gcm2* null) blood calcium declines to the normal adult level (maternal level).

V. Discussion

The present doctoral research examined the role of PTH in fetal-placental calcium and bone metabolism because the role of PTH in regulating these processes was uncertain. Based on the published findings in the *Hoxa3* null fetal mice and other data reviewed in the introduction, it was hypothesized that: *PTH, despite its low circulating levels during fetal life, plays an important role in regulating not only fetal blood calcium and skeletal development, but also placental calcium transfer.*

Two different gene knockout mouse models within the same Black Swiss genetic background were utilized: the *Pth* null, which served as a model of complete absence of PTH, and the *Gcm2* null, which served as a model of severe hypoparthyroidism (i.e. significantly reduced PTH). The phenotypes of the two nulls were found to be quite similar, except that placental calcium transfer was increased in *Gcm2* nulls but unchanged in *Pth* nulls. In addition, calcitropic gene expression within the placenta differed between the two nulls, and treating the *Pth* nulls with exogenous PTH elicited an exciting finding: placental calcium transfer increased.

The following discussion will cover the current findings and highlight the role of PTH in regulating fetal blood calcium, fetal skeletal development, and in greater depth, placental calcium transfer and placental gene expression.

A. Is the Phenotype of the *Pth* Null Fetal Mice in the Black Swiss Background the Same as the *Pth* Null Fetal Mice in the C57BL/6 Background? *No*

The present study showed that the *Pth* null fetuses had a mild hypoparathyroid phenotype, with blood calcium reduced only modestly (equal to the maternal level), hyperphosphatemia, low amniotic fluid calcium and magnesium content, and a reduction in the skeletal mineral content. Similarly, *Gcm2* null fetuses had reduced serum PTH levels and a very similar phenotype to that of the *Pth* null fetuses. Furthermore, other than being undermineralized, the skeletons of both *Gcm2* null and *Pth* null fetuses were normal. That is, the skeletal scaffold of the fetuses developed normally, showing no severe abnormalities in endochondral bone formation, limb length, cellular morphology, or mineralization pattern. These results are in contrast to those previously published on the *Pth* null mice. Originally, *Pth* nulls were reported to have numerous skeletal abnormalities including slightly shortened long bones, tibial diaphysis that were significantly shorter than normal, enlarged hypertrophic zones and decreased osteoblast number (73) (For a summary of present and previous findings see **Table 4**). The discrepancy between the present and the previous study may be attributed to differences in genetic background. In the previous study, the *Pth* nulls were studied in a C57BL/6 background, while in the present study the Black Swiss background was utilized. It has previously been shown that Black Swiss fetuses have a higher ionized calcium level than C57BL/6 mice (67), and that the Black Swiss mice have a higher bone mineral density than C57BL/6 mice (data from Kovacs lab, submitted). This and other genetic differences between the Black Swiss and C57BL/6 may impact the overall physiology of the mice

and result in a phenotypic difference. As a result, the skeletal development of the *Pth* null fetal mice was more severely affected in the C57BL/6 background. The idea of genetic background affecting phenotype is consistent with the findings from other studies. For example, in both *Pthr1* null and multiple endocrine neoplasia type 1 (*Men1*) null mice, genetic background was shown to have an affect on lethality. In the hybrid C57BL/6-129/SvJ background, *Pthr1* null mice died at mid-gestation; while in the Black Swiss background they survived until immediately after birth (67). In *Men1* null mice, embryonic lethality was extended by two days when backcrossed into the C57BL/6 background as compared to the 129S6/SvEv background. Genetic background has also been shown to have a dramatic affect on the occurrence of neural tube defects in the *Men1* null fetal mice. In fact, neural tube defects did not occur in *Men1* null mice when placed in the C57BL/6 background, but occurred exclusively when backcrossed into the 129S6/SvEv background (122).

The fact that two independent models of PTH deficiency, studied in the same genetic background, showed reduced blood calcium levels and undermineralized skeletons indicates that PTH is indeed required for the fetus to maintain the normally high blood calcium level that is observed in the fetus (higher than the maternal level), and to sufficiently mineralize the fetal skeleton before birth. Furthermore, contrary to what was previously thought (20), it shows that maintaining a blood calcium at the maternal level is not necessarily sufficient for the fetus to mineralize the fetal skeleton before birth. With the decline in blood calcium to the maternal level, there was a 5-10% reduction in skeletal mineral content in both the *Pth* null and *Gcm2* null fetuses respectively. It is

likely that PTH contributes to skeletal mineralization through maintaining the high blood calcium level that is normally observed in fetuses, which would allow sufficient calcium to be readily available to mineralize newly formed fetal bone before birth. This implies that one purpose of the fetal hypercalcemia may be to facilitate skeletal mineralization.

B. Does Absence of PTH Have the Same Consequences on Skeletal Mineralization and Blood Calcium as Absence of PTH and Parathyroids? If Not, Why Does the Phenotype of the *Pth* Nulls Differ From That of the *Hoxa3* Nulls? *No*

If absence of PTH alone explained the *Hoxa3* null phenotype, then *Pth nulls* and *Hoxa3* nulls should have the same phenotype. However, this is not the case. In previous studies, *Hoxa3* null fetal mice (lacking PTH and parathyroids) were found to have a significantly reduced blood calcium level (well below the maternal level), hyperphosphatemia, hypomagnesemia, low mineral content in the amniotic fluid, and a 25% decrease in skeletal calcium content (72). The more severe phenotype (lower blood calcium and less calcium content of the skeleton) of the *Hoxa3* null fetuses as compared to both *Pth* null and *Gcm2* null fetuses, which had a blood calcium equal to the maternal level and a 14% and 19 % reduction in skeletal calcium content, respectively, confirms that the absence of parathyroids and PTH causes a more severe abnormality than absence of PTH alone (for a summary of previous and present findings see **Table 4**). Furthermore, contrary to what was originally thought, PTH does not have a more dominant effect on fetal blood calcium regulation than PTHrP. In the absence of PTHrP (*Pthrp* null fetuses), absence of PTH (*Pth* null), and reduced PTH (*Gcm2* null) blood calcium declined to the

normal adult level (maternal level). In the complete absence of PTH and parathyroids (*Hoxa3* null fetuses) however, the blood calcium declined to a level below the maternal level. The lowest blood calcium occurred with absence of PTH, PTHrP, and parathyroids (*Pthrp/Hoxa3* null) or absence of the amino-terminal actions of both PTH and PTHrP (*Pth1r* null) **(Figure 34)**.Thus, in the absence of one hormone (PTH or PTHrP), blood calcium is moderately affected and one hormone does not make up for the lack of the other. When both hormones are absent or when the parathyroids are absent, blood calcium is more severely affected.

Why might the absence of PTH and parathyroids affect fetal blood calcium and skeletal mineralization more severely than PTH alone? The fact that the *Pth* null and *Hoxa3* null mice were studied in the same genetic background rules out that the phenotypic differences were due to different genetic background. Rather, it may be that removing the parathyroids causes a greater effect because it not only removes PTH, but also other secretory products of the parathyroids. Among them are endothelin-1 (123), chromogranin A (CgA) (124) and parathyroid hypertensive factor (PHF) (125).

In previous studies endothelin-1 has been shown to stimulate both bone formation and resorption (126) and act as a vasoconstrictor in fetal systemic and umbilical vascular beds (127). The role of CgA has previously been examined by creating a chromogranin A gene (*Chga*) knockout mouse model. In one report, *Chga* nulls had elevated urinary catecholamine excretion, but otherwise seemed normal. They were viable, showed no developmental abnormalities, and locomotor and endocrine functions appeared to be normal including serum PTH, phosphorous, and calcium levels. It was also noted that

other granins were upregulated (e.g. CgB and secretogranin II) in the adrenal medulla, hypothalamus, pituitary and thyroid, and it was concluded that other chromogranins were likely compensating for the lack of CgA (128). In another study, it was reported that some *Chga* null mice died prenatally, and that those born suffered severe growth retardation in the first 4-5 weeks after birth. Furthermore, these mice were hypertensive (129). Lack of CgA may not be solely responsible for the phenotype of *Chga* null mice because secreted CgA is cleaved into numerous biologically active peptides which among other things, inhibit vasoconstriction and inhibit the release of certain hormones (130). PHF, as the name suggests, is associated with some forms of hypertension (131). It has also been shown to increase vascular smooth muscle calcium uptake (131, 132).

It is possible that lacking one or more of parathyroid derived endothelin, CgA, or PHF could account for the more severe phenotype observed in the *Hoxa3* nulls, but whether or not any of these factors actually play a role in fetal calcium and bone metabolism is unknown. Another possibility is that the parathyroids produce some as yet unknown factor that regulates fetal mineral homeostasis. Finally, the *Hoxa3* nulls suffer from other abnormalities, including absence of the thymus and thyroid hypoplasia, which may also somehow contribute to the more severe hypoparathyroid phenotype of *Hoxa3* nulls.

Like the *Hoxa3* nulls the *Gcm2* nulls are also lacking the parathyroid glands, so why is the phenotype in the *Gcm2* nulls equivalent to the *Pth* nulls, and not as severe as the *Hoxa3* nulls? The fact that the *Gcm2* nulls have some PTH and/or the fact that they can increase placental calcium transfer, may allow them to compensate for the lack of

other parathyroid derived factors (e.g. CgA, PHF), whereas the *Hoxa3* nulls cannot. Alternatively, if there is a small cluster of cells in the thymus of *Gcm2* nulls that produce some PTH, as was suggested by Gunther *et al.* (2000), then perhaps those cells can also produce the other parathyroid derived factors. However, the work from Dr. Nancy Manley's lab, cited earlier, makes it unlikely that Gunthers hypothesis is correct.

C. Does PTHrP Increase in Response to Fetal Hypocalcemia and/or Absence of PTH? *No*

In the present study, although both the *Pth* nulls and *Gcm2* nulls experienced the same degree of hypocalcemia and a similar skeletal phenotype, only the *Gcm2* nulls show a small, but significant increase in placental calcium transfer. It was hypothesized that the small amount of PTH in the *Gcm2* nulls could be responsible for stimulating placental calcium transfer above the normal WT value. However, because PTHrP is known to regulate placental calcium transfer (67-70), PTHrP was assessed to determine if it could account for the increase in placental calcium transfer seen in the *Gcm2* null fetuses. It was found that both nulls had normal plasma PTHrP levels.

Next, we evaluated PTHrP expression in the neck region (containing fetal parathyroids) of both nulls. Admittedly, it is controversial as to weather or not murine fetal parathyroids produce PTHrP in addition to PTH (20). While there is evidence that fetal sheep parathyroids produce PTHrP (70), a careful study of fetal rat parathyroids has shown no evidence of PTHrP mRNA by RT-PCR (114). The whole neck region was used because the fetal parathyroids are very small and difficult to isolate. As assessed by

quantitative real-time RT-PCR, PTHrP mRNA expression in the neck region of both *Pth* null and *Gcm2* null fetuses was not increased. It is possible that the tissue studied (neck section) was too large, and obscured the PTHrP signal from the parathyroids. But given the normal circulating level of PTHrP, it seems unlikely that the parathyroids could be producing an increased amount of PTHrP that would have to circulate in order for it to act on the placenta, and that it would not have be detected in the circulation.

In addition to working in an endocrine manner, PTHrP can also function in an autocrine/paracrine fashion. Thus, PTHrP may have only increased locally in the placenta to stimulate placental calcium transfer. In both the *Gcm2* nulls and *Pth* nulls however, there was no upregulation of PTHrP mRNA observed in the placenta. In combination, the lack of increase in plasma PTHrP, and lack of increase in PTHrP mRNA in the neck or placenta, makes it unlikely that PTHrP is responsible for the increase in placental calcium transfer observed in the *Gcm2* null fetuses.

The phenotype of both the *Pth* null and *Gcm2* null fetuses (although not as severe as the *Hoxa3* nulls) was similar to that of the *Hoxa3* null fetuses, which also did not upregulate PTHrP despite having an even lower blood calcium level as compared to both *Pth* and *Gcm2* null fetuses (72). Thus, it is evident from the previous and present study that PTHrP does not increase in response to fetal hypocalcemia and/or absence of PTH. This is in contrast to PTH which has been shown to increase in response to fetal hypocalcemia caused by numerous factors including maternal hypocalcemia (31), deletion of the *Pthrp* gene (29), and inactivating mutations of the CaSR (15). Thus, in fetal life, it appears that PTH and PTHrP are regulated differently. Because PTH

increases in response to fetal hypocalcemia, it is likely that PTH is regulated by the CaSR on the parathyroids and normally kept suppressed by the high fetal blood calcium. PTHrP however, does not appear to be regulated by the CaSR or blood calcium. Instead, it may be regulated by other factors, such as the amount of calcium that is being transferred across the trophoblasts and the IPYS (20). Alternatively, it is possible that PTHrP is autonomously produced by the placenta and not subject to any regulation by calcium flux.

D. Does PTH Regulate Placental Calcium Transfer? *Yes*

Over the years many studies have attempted to explain the process of placental calcium transfer and the influential factors that affect it. These studies have utilized various techniques for assessing the transfer of calcium across the placenta, namely the placental perfusion technique (94, 95, 98, 99) and a technique for assessing intact fetal mice (15, 33, 67, 72). There are inconsistencies among various studies, but strong experimental evidence has led to the consensus that PTHrP (the mid-molecular region) regulates placental calcium transfer (67-70).

There is less consistent evidence for a role of PTH in regulating placental calcium transfer. Thyroparathyrodectomized fetal sheep were reported to have reduced placental calcium transfer, which remained unaltered when the placenta was infused with PTH (as assessed by placental perfusion) (70). Similarly, reduced placental calcium transfer in the *Pthrp* null fetal mice was also found to be unaffected by *in utero* treatments of PTH (as assessed with the fetus intact) (67). In addition, it was reported that *Hoxa3* null fetuses

have normal placental calcium transfer, despite the completely absence of PTH (72). On the other hand, studies in decapitated (to simulate a parathyroidectomy) fetal rats, showed that PTH [1-84] increased the rate of placental calcium transfer (96). Furthermore, PTH [1-34] has been shown to increase calcium transport across vesicles that were created *in vitro* from human syncytiotrophoblast basal membranes (115).

In the present study *Gcm2* nulls, which have some PTH, showed an increase in placental calcium transfer. Since PTHrP was not increased in the circulation, neck or placenta of *Gcm2* nulls, we hypothesized that PTH may explain the increase in placental calcium transfer. We directly tested this by treating *Pth* null fetuses *in utero* with either saline or PTH [1-84], and then performing the placental calcium transfer experiment. A statistically significant increase in placental calcium transfer was observed in the *Pth* null fetuses treated with PTH [1-84], as compared to *Pth* null fetuses treated with saline. This data provides more substantial evidence to support the thesis that: *PTH, despite its low circulating levels during fetal life, plays an important role in regulating not only fetal blood calcium and skeletal development, but also placental calcium transfer.*

Based on the previously discussed *in vitro* studies on human vesicles created from human placental cells, and the fact that the PTH1R is highly expressed in the placenta, particularly in the IPYS which is believed to be a site of maternal-fetal calcium exchange (92), it is likely that PTH[1-84] exerts its effects on placental calcium transfer by binding to the PTH1R. This is because the PTH1R binds PTH [1-34], but not the mid-molecular region of PTHrP which is known to regulate placental calcium transfer.

As previously discussed, a strong piece of evidence that supported the notion that PTH does not regulate placental calcium transfer is that treating *Pthrp* null fetuses (that have reduced placental calcium transfer) with mid-molecular fragments of PTHrP increased placental calcium transfer, while treatment with PTH [1-84] did not (67). The current findings however, suggest that PTH [1-84] can stimulate placental calcium transfer. In retrospect, *Pthrp* null fetuses were found to have a significant increase (approximately 3 fold versus WT) in endogenous PTH (29), and this may explain why *Pthrp* null fetuses failed to respond to PTH treatment in the earlier experiments.

E. Does PTH Regulate Placental Gene Expression? *Yes*

In light of the exciting finding that treating *Pth* null fetuses *in utero* with PTH [1-84] increased placental calcium transfer, the next step was to explore the possible mechanisms by which PTH might be acting to regulate this process. Initially, gene expression at baseline (placenta from *Pth* nulls treated *in utero* with saline verses placenta from WTs treated *in utero* with saline) was examined. As assessed by microarray and real-time quantitative RT-PCR, it was found that at baseline, *Pth* null placentas had a significant decrease in *Trpv6*, *Cabp9k*, and *Vdr* mRNA's as compared to WT placenta. Furthermore, the microarray showed that the expression of several other genes involved in cation and solute transport were significantly decreased. The fact that all of these genes were significantly downregulated in the *Pth* null placenta as compared to WT suggests that PTH may regulate not only calcium, but other solute transfer. Furthermore, the fact that *Trpv6*, *Cabp9k*, and *Vdr* were all downregulated in the *Pth* null

placenta, at first glance it would seem to predict that placental calcium transfer would also be decreased in the *Pth* null mice. This is not the case however; placental calcium transfer was normal in the *Pth* null fetuses. This may be explained by the findings from previous studies. In *Trpv6* null mice, placental calcium transfer was decreased (93), and in *Pthrp* nulls, which have reduced placental calcium transfer, the expression of CaBP9k in the IPYS was significantly reduced (67, 92). Placental calcium transfer of *Vdr* nulls however, was reported to be increased, along with the placental expression of TRPV6 (33), indicating that VDR may act as a break on the placental calcium transfer system, such that in its absence, placental calcium transfer increases. Thus, in the present study the relative changes in TRPV6, CaBP9k, and VDR, may have balanced each other out, leading to no net change in placental calcium transfer.

Next, changes in gene expression in response to PTH treatment were examined between placentas from *Pth* nulls treated *in utero* with PTH [1-84] versus placentas from *Pth* nulls treated *in utero* with saline. It was found that the expression of *Vdr* and vitamin D binding protein increased significantly, along with several solute carriers, when fetuses were treated with PTH [1-84] as compared to saline. These samples were collected at the same time point as when placental calcium transfer was assessed; 90 min after the *in utero* saline or PTH [1-84] injections. With this short interval, it is unlikely that the changes observed in mRNA expression could result in protein synthesis occurring fast enough to account for the differences in placental calcium transfer that was observed. Rather, it is likely that PTH achieved its effect on placental calcium transfer by working directly on its receptor in the placenta to open channels to allow more calcium to be

transferred from mother to fetus. However, observing changes in mRNA expression at 90 minutes shows that PTH can indeed regulate placental calcitropic gene, and other solute transporter, expression.

Although *Gcm2* null placentas had significantly lower expression of both *Trpv6* and *Cabp9k* as compared to WT (like *Pth* nulls), and *Vdr* expression no different from that of WT, when placental gene expression between the *Pth* nulls and *Gcm2* nulls (no treatment) was compared, it was revealed that the expression of *Trpv6*, *Cabp9k*, and *Vdr* were all significantly increased in the *Gcm2* null placentas as compared to *Pth* null placentas. This may in part explain the difference in placental calcium transfer observed between the *Pth* nulls and *Gcm2* nulls. More specifically, why placenta calcium transfer was increased in the *Gcm2* null placentas, but not in the *Pth* null placentas despite having a similar biochemical phenotype.

F. Is PTH Expressed Locally in the Placenta? *Yes*

The finding that PTH can regulate placental gene expression prompted the consideration that PTH might be expressed in the placenta. As assessed by real-time quantitative RT-PCR, it was discovered that PTH was present in the placenta of WT and both *Pth* null and *Gcm2* null fetuses. Furthermore, PTH was found to be significantly higher in the *Gcm2* null placenta as compared to the *Pth* null placenta. Thus, it is likely that the low circulating levels of PTH in the *Gcm2* null fetuses is coming from not only the thymus (if at all), but also the placenta. It may be the case that PTH acts locally in the placenta to regulate calcium, and other solute transfer, and that the circulating PTH may,

or may not be relevant. Based on these and the previously discussed findings, although it has not been directly tested in the present study, it is conceivable that PTH in *Gcm2* null fetuses plays a role in regulating placental calcium transfer.

PTH was significantly lower in *Pth* null placenta as compared to both WT and *Gcm2* null placenta, but it was still detectable. This may reflect a false positive expression finding, or it may be that the small amount of PTH that was detected in the *Pth* null placenta was derived from the maternally derived tissue portion of the placenta. To determine the source of the PTH production in the placenta *in situ* RT-PCR and/or immunohistochemistry could be utilized. Alternatively, it would be interesting to study PTH expression in placentas collected from *Pth* nulls from a *Pth* null pregnancy to determine if the signal is eliminated.

If PTH is expressed locally in the placenta and is contributing to the increase in calcium transfer observed in the *Gcm2* null fetuses, the question remains why the *Hoxa3* nulls do not increase calcium transfer in response to severe hypocalcemia? They, like the *Gcm2* nulls, lack the parathyroids, but presumably are still capable of producing PTH. Although it remains to be investigated, it is speculated that deleting the *Hoxa3* gene not only affects the development of the tissues deriving from the third and fourth pharyngeal pouch, but for some yet unknown reason also affects placental morphology and/or placental gene expression which results in the *Hoxa3* null placenta not being able to produce PTH locally and/or increase calcium transfer.

G. Study Pitfalls and Limitations

An unresolved inconsistency in the present study is that although placental calcium transfer was not reduced in either the *Gcm2* null or *Pth* null fetuses, both nulls had reduced blood calcium, reduced amniotic fluid calcium and reduced skeletal calcium content. One explanation is that backflux, or flow of calcium from fetus to mother may have been increased in order to account for the lack of mineral present in the fetuses. The technique used in this study to assess placental calcium transfer does not measure backflux. However, the short 5 minute interval used between administering the isotopes and the removal of the fetus makes it less likely that the placental calcium transfer measurement is not confounded by backflux of the isotope. This is evident from previous studies on *Pthrp* knockout mice, which reported that extending the time between isotope administration and removal of the fetuses increased the relative difference in placental calcium transfer between the genotypes, which likely indicates that the longer the interval, the more the results are confounded by backflux. Thus, with only the 5 minute interval there is less time for the isotope to be transferred from the mother to the fetus, become diluted in the circulation of the fetus, and return in substantial amounts across the placenta to the mother (67). Alternatively, the fact that placental calcium transfer was not reduced in either the *Gcm2* null or *Pth* null fetuses, but that both nulls had reduced blood calcium, reduced amniotic fluid calcium and reduced skeletal calcium content, may indicate that the "normal" (i.e. WT) rate of calcium transfer is only adequate if the parathyroids and normal PTH levels are present. Thus, without normal PTH levels and/or

parathyroids the "normal" rate of calcium transfer is not sufficient to meet the demands of the developing fetus.

Another pitfall in the present study is that neither *Pth* null nor *Gcm2* null placenta morphology was assessed as compared to WT. Thus, it remains possible that placental calcium transfer in either the *Pth* nulls and/or *Gcm2* nulls was affected by morphological changes in the placenta.

Animal research is important in order to uncover the complexity of living systems, and understand bodily processes, diseases and possible treatments. However, as with any research, there are limitations. The present work showed that deficiency of PTH in fetal life, results in hypocalcemia and an undermineralization skeleton, and that PTH plays a role in regulating placental calcium transfer and can affect placental gene expression. Although insightful, the limitations of extrapolating these findings and applying them to humans must be addressed.

Mice are great experimental models to use when studying placental function, because the overall structure of human and rodent placenta are similar, with both being discoid and haemochorial. However, several structural differences exist between the rodent and human placenta. First, rodents have a haemotrichorial placenta, while humans have a haemomonochorial placenta (89). Second, rodents have an intricate labyrinth where the maternal and fetal blood comes in close contact with one another, whereas in humans villi from the fetal side protrude into the intervillous maternal blood-filled space and float freely (91). Finally, unlike humans, the rodent placenta contains an IPYS which is believed to be a site of maternal-fetal calcium exchange (20, 92, 93). As a result of

these structural differences, it is possible that the human and rodent placenta do not function similarly. However, whether these structural differences result in functional differences or not remains unknown. It may also be the case that differences exist simply due to difference between species. Thus, our findings in mice may not necessarily be applicable to humans.

In the present study, knockout mouse models allowed us to explore the role of PTH in fetal-placental calcium and bone homeostasis, in a way that would obviously not be suitable if dealing with humans. Although useful, there are limitations when using such models. One notable challenge is that of compensatory effects. That is, when knocking out a gene of interest it is possible that the effect observed, or lack thereof, may be the result of another hormone or mechanism being triggered that compensates for the lack of the gene of interest. Also, when knocking out a gene, it is possible that the phenotype observed may not be directly due to the gene knocked out, but rather due to effects on another gene. Consequently, it is clear that one must be cautious in drawing conclusions from studies of single knockout mouse models. In the area of fetal calcium and bone metabolism, much of what we do understand has been drawn from comparing phenotypes of various knockout mouse models. This approach is very useful, but if utilized, the limitations must be acknowledged. That way, studies can be designed in the best possible way to help minimize being led astray. For example, it is necessary to consider genetic background. When making comparisons between knockouts, the genetic background should be the same because phenotypic differences may simply result from the variability in the fetal milieu caused by genetic background.

H. Clinical Implications

The fetal period is known to be a time of rapid skeletal development and mineralization, with 80% of the mineral required before birth being accumulated in the last trimester of pregnancy in humans (7). Elucidating the mechanisms by which the fetus accomplishes this may ultimately lead to novel approaches to treat such conditions as osteoporosis and other metabolic bone diseases.

In humans, fetal hypoparathyroidism may occur for numerous reasons including lack of parathyroid development, as in DiGeorge syndrome (133), or impaired parathyroid development from mutations in the *Gcm2* gene (134-137). The effect that this has on fetal blood calcium, and skeletal development and mineralization however, to my knowledge, is unknown. From these studies it is predicted that PTH deficiency in the fetus will result in hypocalcemia and impaired skeletal mineralization. Knowing this could affect how infants born with hypoparathyroidism are assessed and treated after birth, and how they will be monitored as they age. Furthermore, knowing that such complications could result if PTH deficiency is present *in utero,* could possibly lead to *in utero* treatments to diminish, or avoid, adverse outcomes.

I. Overall Summary and Conclusions

The purpose of the present PhD dissertation was to determine whether PTH is required for regulating fetal-placental mineral homeostasis and skeletal development. It was hypothesized that: *PTH, despite its low circulating levels during fetal life, plays an important role in regulating not only fetal blood calcium and skeletal development, but*

also placental calcium transfer. To address this, two different genetic mouse models were utilized; the *Pth* null mice, which served as a model of complete absence of PTH, and the *Gcm2* null mice which served as a model of severe hypoparathyroidism. The hypothesis was supported as it was determined that in the absence, or deficiency, of PTH a fetal hypoparathyroid phenotype resulted. The fetuses experienced hypocalcemia, hypomagnesemia, hyperphosphatemia, low amniotic fluid mineral content, and reduced skeletal mineral content. Furthermore, PTH was found to be expressed in the placenta, regulated placental expression of genes involved in calcium transfer, including TRPV6 and CaBP9k, and directly stimulated placental calcium transfer. It is though that PTH may contribute to placental gene expression and function, via two different pathways. That is, the endocrine/systemic pathway, which would utilize parathyroid-derived PTH, and the paracrine/autocrine pathway, which would utilize placental-derived PTH. Whether these current findings are applicable to human fetuses that are deficient in (or completely lacking) PTH remains unknown.

VI. Future Directions

The current study presents some very interesting findings regarding the role of PTH in fetal-placental calcium and bone homeostasis. However, many questions still need to be addressed if the importance of PTH in fetal skeletal development and placental function, and how it absence (or deficiency) affects the fetal milieu, is to be completely understood.

First, as the present study suggests that PTH plays a role in regulating placental calcium transfer, it would be of great value to determine which region of PTH is responsible for this process. It is likely that it is the amino-terminal fragment. This is based on the fact that it is the amino-terminal region of PTH that binds to the PTH1R, which is highly expressed in the placenta (and IPYS), and not the receptor that the mid-molecular region of PTHrP functions to regulate placental calcium transfer. Furthermore, in previous studies it was reported that PTH [1-34], the amino-terminal region, increased calcium transport in vesicles that were created from human syncytiotrophoblast basal membranes (115). However, although likely, it remains possible that some other region of PTH, other than the amino-terminal region, is responsible for stimulating placental calcium transfer in mice, and that its actions are mediated via a receptor other than the PTH1R. Thus, this question must be directly addressed. Similar to previous studies in *Pthrp* null mice which showed that it was the mid-molecular region of PTHrP that was responsible for stimulating placental calcium transfer (67), various fragments of PTH could be tested to determine which region of PTH is responsible for stimulating placental calcium transfer.

Second, another very interesting finding in the present study is that PTH was expressed in the placenta. In fact, PTH was not only expressed in the WT placenta, but also (at significantly lower levels) in the placenta of *Pth* nulls. Because the *Pth* nulls cannot produce PTH, it is likely that the PTH detected in the placenta harvested from *Pth* null fetuses was coming from the maternally derived tissue portion of the placenta, which makes up very little of the total placenta (unpublished). In the WT placenta however, it is likely that PTH is expressed in the whole placenta (i.e. placental tissue derived from both maternal and fetal sources) because the expression of PTH is significantly higher in WT placenta as compared to that of *Pth* null placenta. Thus from the current findings it is evident that PTH is locally produced in the placenta, however, where it is produced remains unknown. This is necessary to determine if the role of PTH in fetal-placental calcium and bone homeostasis is to be completely understood. Due to the relatively low expression levels of PTH in the placenta, it was unknown whether PTH in the placenta would be detectable by traditional reverse-transcriptase PCR (RT-PCR). Preliminary studies were carried out, and PTH was found to be detectable in both WT and *Pth* null placenta by RT-PCR, and like quantitative real-time RT-PCR, PTH expression in the *Pth*-null placenta was lower, yet still detectable, as compared to WT placenta (unpublished data). Therefore, although the expression is relatively low, *in situ* RT-PCR could be utilized to localize PTH expression in the placenta.

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VII: Appendicies

APPENDIX A

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APPENDIX C

N - Terminal PTHrP Radioimmunoassay (RIA) Protocol

Reagents

Standard:	Recombinant PTHrP (1-84), reconstitute 2 µg/10µl aliquot in 1 ml assay buffer which gives a concentration of 200,000 pmol/L. Several dilutions ranging from 0.8 to 400 pmol/L concentrations are then made. They included the following standard concentrations: 0, 0.8, 2, 4, 8, 15, 20, 30, 40, 80, 200, 400 pmol/L.
Antibody:	INCSRAR antihuman PTHrP (1-40) goat number 906. Dilute 1/100 stock solution to 1/10, 000 and use 100 µl/tube. Final dilution 1/40,000.
Tracer:	^{125}I Tyr^{0}PTHrP (1-34), 10,000 cpm in 100 µl.
Assay Buffer:	0.05 M Barbitone pH 8.6 with 0.5% BSA, 0.02% Tween, 0.05% Sodium Azide, 0.5 ml (20TIU) Aprotinin (Trasylol)/100 ml assay buffer.
Antigoat Sac Cel:	SAC-CEL Anti-sheep/goat code: AA-SAC2 (Science Made Simple [IDS]).
Aprotinin:	Protease inhibitor from bovine lung (Sigma A-1153), reconstitute 100 mg with 10 ml 0.05M barbital buffer, pH 8.6.
Plasma samples:	Collect in 0.5 M EDTA 100 µl (or EDTA coated tube) and Aprotinin 100 µl (4TIU)/10 ml of blood.
Pooled normal plasma:	EDTA plasma with Aprotinin from haemochromatosis patients are pooled and used as control for binding changes observed in the presence of plasma. 100 µl is added to each standard tube.
Controls C1, C2 and C3:	Low, medium and high levels (C1, C2 and C3) of recombinant human PTHrP (1-84) are added to normal human plasma.

Assay Procedure

Day 1:

Incubate the following: Standard or sample (50 µl)
Antibody (50 µl)
Assay buffer/pooled normal plasma (50 µl)

Non-specific binding tube, incubate the following: Assay buffer (100 µl)
Normal plasma (50 µl)
At room temperature for 4 hours

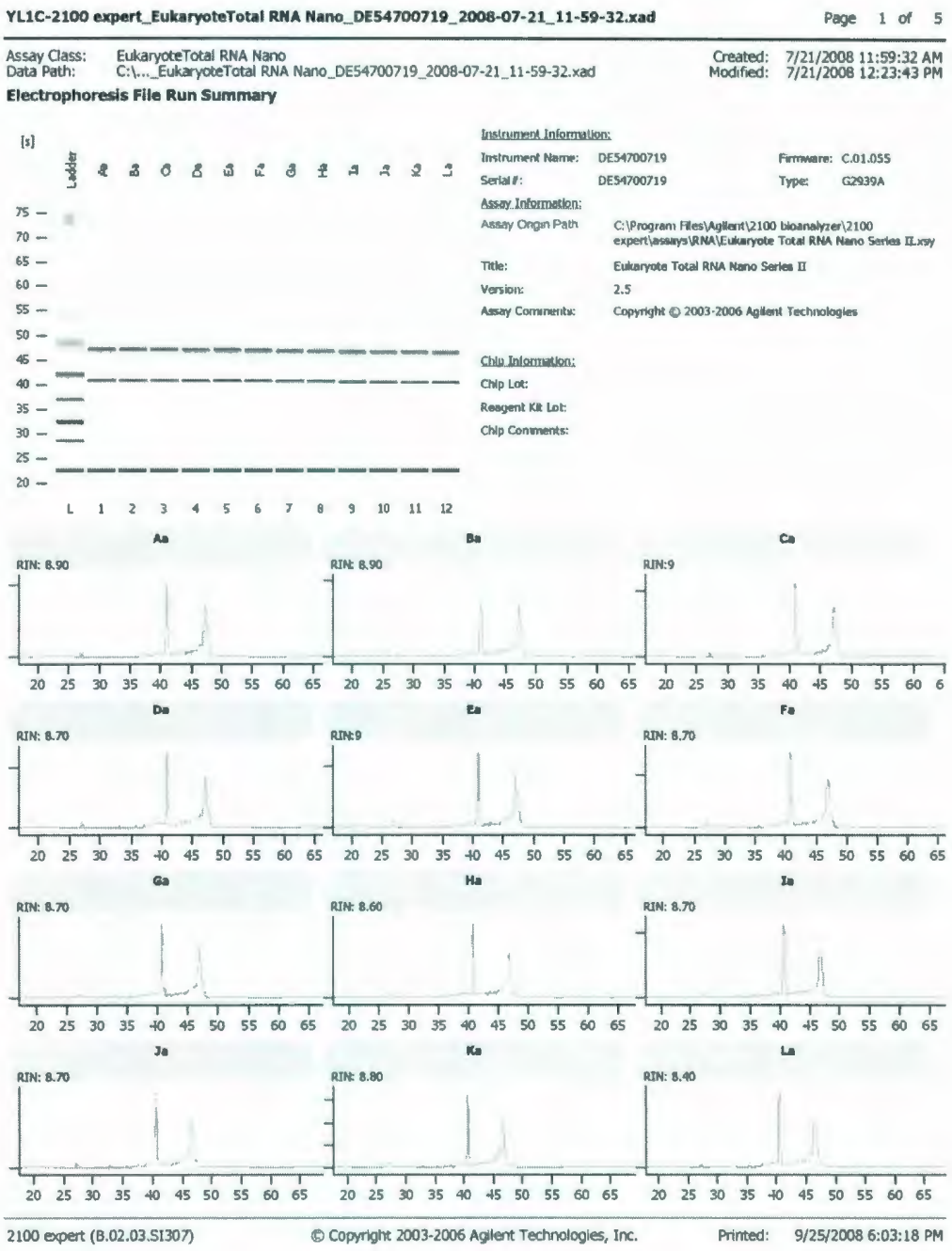
Add tracer, 5000 cpm (50 µl) and incubate overnight at room temperature.
Final incubation volume is 200 µl per tube.

Day 2:

Add anti-goat Sac-Cel (50µl) and incubate for 30 minutes at room temperature.
Add 0.5 ml distilled water to each tube.
Centrifuge and aspirate supernatant.
Count pellet using multigammer counter (Cobra 5005, Packard, Downer Grover, IL)

APPENDIX D

Bioanalyser Results



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Modified: 7/21/2008 12:23:43 PM

Electrophoresis File Run Summary (Chip Summary)

Sample Name	Sample Comment	Status	Observation	Result Label	Result Color
Aa		✓		RIN: 8.90	
Ba		✓		RIN: 8.90	
Ca		✓		RIN:9	
Da		✓		RIN: 8.70	
Ea		✓		RIN:9	
Fa		✓		RIN: 8.70	
Ga		✓		RIN: 8.70	
Ha		✓		RIN: 8.60	
Ia		✓		RIN: 8.70	
Ja		✓		RIN: 8.70	
Ka		✓		RIN: 8.80	
La		✓		RIN: 8.40	

Chip Lot # **Reagent Kit Lot #**

Chip Comments :

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Data Path: C:\..._EukaryoteTotal RNA Nano_DE54700719_2008-07-21_11-59-32.xad

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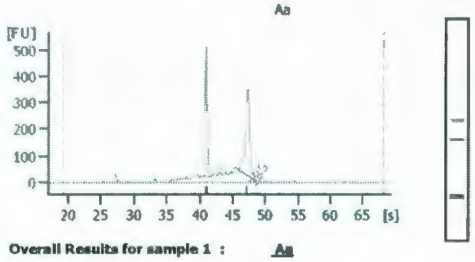
Modified: 7/21/2008 12:23:43 PM

Electropherogram Summary

Aa



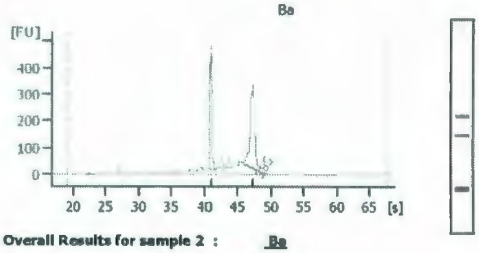
Overall Results for sample 1 : Aa

RNA Area:	1,782.9
RNA Concentration:	1,921 ng/µl
rRNA Ratio [28s / 18s]:	1.3
RNA Integrity Number (RIN):	8.9 (B.02.03)
Result Flagging Color:	
Result Flagging Label:	RIN: 8.90

Fragment table for sample 1 : Aa

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	40.66	41.64	402.1	22.6
28S	45.84	48.61	541.1	30.4

Ba



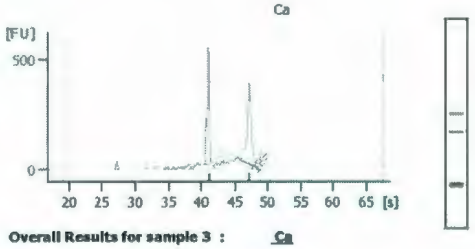
Overall Results for sample 2 : Ba

RNA Area:	1,650.8
RNA Concentration:	1,779 ng/µl
rRNA Ratio [28s / 18s]:	1.3
RNA Integrity Number (RIN):	8.9 (B.02.03)
Result Flagging Color:	
Result Flagging Label:	RIN: 8.90

Fragment table for sample 2 : Ba

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	40.63	41.61	378.3	22.9
28S	45.84	48.68	500.0	30.3

Ca



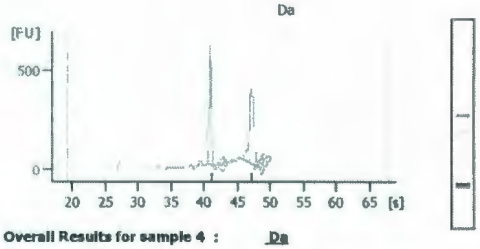
Overall Results for sample 3 : Ca

RNA Area:	1,924.0
RNA Concentration:	2,073 ng/µl
rRNA Ratio [28s / 18s]:	1.3
RNA Integrity Number (RIN):	9.0 (B.02.03)
Result Flagging Color:	
Result Flagging Label:	RIN:9

Fragment table for sample 3 : Ca

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	40.64	41.62	445.4	23.1
28S	45.78	48.63	600.2	31.2

Da



Overall Results for sample 4 : Da

RNA Area:	2,211.4
RNA Concentration:	2,383 ng/µl
rRNA Ratio [28s / 18s]:	1.3
RNA Integrity Number (RIN):	8.7 (B.02.03)
Result Flagging Color:	
Result Flagging Label:	RIN: 8.70

Fragment table for sample 4 : Da

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	40.57	41.55	490.9	22.2
28S	45.69	48.59	635.0	28.7

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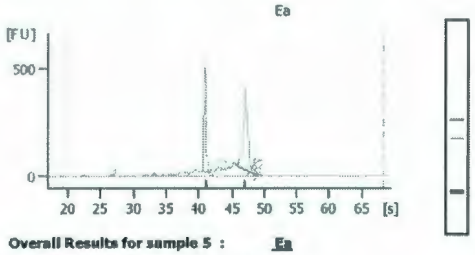
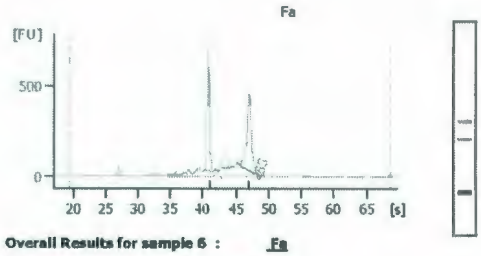


Overall Results for sample 5 : Ea

RNA Area:	2,049.9
RNA Concentration:	2,209 ng/µl
rRNA Ratio [28s / 18s]:	1.3
RNA Integrity Number (RIN):	9.0 (B.02.03)
Result Flagging Color:	
Result Flagging Label:	RIN:9

Fragment table for sample 5 : Ea

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	40.59	41.58	478.2	23.3
28S	45.69	48.62	636.4	31.0

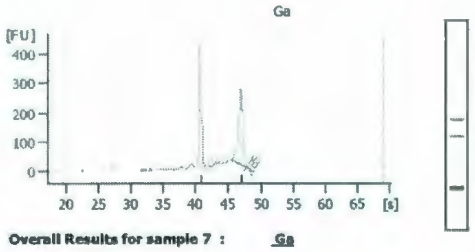


Overall Results for sample 6 : Fa

RNA Area:	2,594.3
RNA Concentration:	2,795 ng/µl
rRNA Ratio [28s / 18s]:	1.3
RNA Integrity Number (RIN):	8.7 (B.02.03)
Result Flagging Color:	
Result Flagging Label:	RIN: 8.70

Fragment table for sample 6 : Fa

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	40.52	41.47	573.6	22.1
28S	45.60	48.48	745.2	28.7

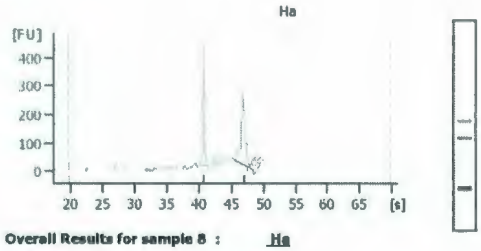


Overall Results for sample 7 : Ga

RNA Area:	1,493.4
RNA Concentration:	1,609 ng/µl
rRNA Ratio [28s / 18s]:	1.3
RNA Integrity Number (RIN):	8.7 (B.02.03)
Result Flagging Color:	
Result Flagging Label:	RIN: 8.70

Fragment table for sample 7 : Ga

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	40.44	41.39	330.2	22.1
28S	45.50	48.36	428.1	28.7



Overall Results for sample 8 : Ha

RNA Area:	1,548.1
RNA Concentration:	1,668 ng/µl
rRNA Ratio [28s / 18s]:	1.3
RNA Integrity Number (RIN):	8.6 (B.02.03)
Result Flagging Color:	
Result Flagging Label:	RIN: 8.60

Fragment table for sample 8 : Ha

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	40.40	41.36	335.7	21.7
28S	45.51	48.34	422.5	27.3

Assay Class: EukaryoteTotal RNA Nano
Data Path: C:\..._EukaryoteTotal RNA Nano_DE54700719_2008-07-21_11-59-32.xad

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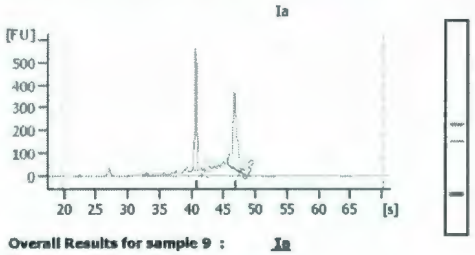
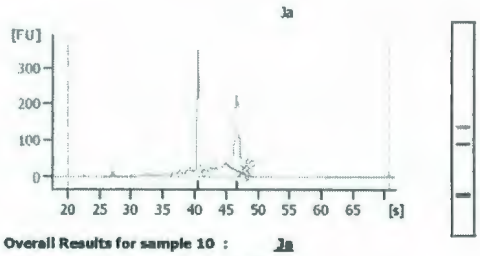


Overall Results for sample 9 : Ia

RNA Area:	2,086.3
RNA Concentration:	2,248 ng/µl
rRNA Ratio [28s / 18s]:	1.3
RNA Integrity Number (RIN):	8.7 (B.02.03)
Result Flagging Color:	
Result Flagging Label:	RIN: 8.70

Fragment table for sample 9 : Ia

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	40.32	41.28	457.3	21.9
28S	45.31	48.21	601.6	28.8

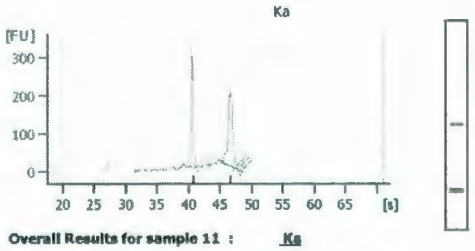


Overall Results for sample 10 : Ja

RNA Area:	1,258.1
RNA Concentration:	1,355 ng/µl
rRNA Ratio [28s / 18s]:	1.2
RNA Integrity Number (RIN):	8.7 (B.02.03)
Result Flagging Color:	
Result Flagging Label:	RIN: 8.70

Fragment table for sample 10 : Ja

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	39.93	41.26	283.9	22.6
28S	45.26	48.18	351.5	27.9

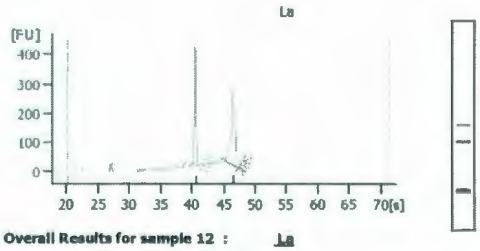


Overall Results for sample 11 : Ka

RNA Area:	1,128.1
RNA Concentration:	1,215 ng/µl
rRNA Ratio [28s / 18s]:	1.3
RNA Integrity Number (RIN):	8.8 (B.02.03)
Result Flagging Color:	
Result Flagging Label:	RIN: 8.80

Fragment table for sample 11 : Ka

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	40.21	41.19	250.4	22.2
28S	45.21	48.14	327.3	29.0



Overall Results for sample 12 : La

RNA Area:	1,577.7
RNA Concentration:	1,700 ng/µl
rRNA Ratio [28s / 18s]:	1.3
RNA Integrity Number (RIN):	8.4 (B.02.03)
Result Flagging Color:	
Result Flagging Label:	RIN: 8.40

Fragment table for sample 12 : La

Name	Start Time [s]	End Time [s]	Area	% of total Area
18S	40.24	41.17	330.6	21.0
28S	45.21	48.10	425.9	27.0

APPENDIX E

Journal of Bone and Mineral Research Publication

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ORIGINAL ARTICLE

JBMR

Parathyroid Hormone Regulates Fetal-Placental Mineral Homeostasis

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ABSTRACT

Parathyroid hormone (PTH) plays an essential role in regulating calcium and bone homeostasis in the adult, but whether PTH is required at all for regulating fetal-placental mineral homeostasis and skeletal development is uncertain. We hypothesized that despite its low circulating levels during fetal life, PTH plays a critical role in regulating these processes. To address this, we examined two different genetic models of PTH deficiency. *Pth* null mice have enlarged parathyroids that are incapable of making PTH, whereas *Gcm2* null mice lack parathyroids but have PTH that arises from the thymus. *Pth* nulls served as a model of complete absence of PTH, whereas *Gcm2* nulls were a model of severe hypoparathyroidism. We determined that PTH contributes importantly to fetal mineral homeostasis because in its absence a fetal hypoparathyroid phenotype results with hypocalcemia, hypomagnesemia, hyperphosphatemia, low amniotic fluid mineral content, and reduced skeletal mineral content. We also determined that PTH is expressed in the placenta, regulates the placental expression of genes involved in calcium and other solute transfer, and may directly stimulate placental calcium transfer. Although parathyroid hormone–related protein (PTHrP) acts in concert with PTH to regulate fetal mineral homeostasis and placental calcium transfer, unlike PTH, it does not upregulate in response to fetal hypocalcemia. © 2010 American Society for Bone and Mineral Research.

KEY WORDS: FETUS; PTH/PTHRP; BONE MINERALIZATION; ION TRANSPORT/PLACENTA; KNOCKOUT; ANIMAL MODELS/RODENT; GROWTH AND DEVELOPMENT

Introduction

Parathyroid hormone (PTH) plays an essential role in regulating calcium and bone homeostasis in the adult. Absence or peripheral resistance to its actions causes hypocalcemia, hyperphosphatemia, reduced bone turnover, and calcifications of soft tissues and basal ganglia.[1]

In contrast, whether PTH is required at all for regulating fetal-placental mineral homeostasis and skeletal development is uncertain. The fetal biochemical milieu differs from the adult and is characterized by elevations of ionized calcium, magnesium, and phosphate above the maternal values and active transfer of calcium, magnesium, and phosphate across the placenta.[2] The skeleton rapidly mineralizes during the third trimester in humans and the last 4 to 5 days of gestation in rodents.[2] Circulating levels of PTH and 1,25-dihydroxyvitamin D (calcitriol) are low compared with adult values, whereas parathyroid hormone–related protein (PTHrP) is increased in the fetal circulation during late gestation.[3] These and other observations prompted the hypothesis that PTHrP might assume the actions of PTH during fetal life, and our previous studies of fetal mineral homeostasis in *Pthrp* null fetuses examined this possibility.

We found that *Pthrp* null fetuses had hypocalcemia (equal to maternal blood level), hypomagnesemia, hyperphosphatemia, reduced fetal-placental calcium transfer, normal amniotic fluid calcium content, and normal skeletal mineral content.[4,5] Serum PTH was increased threefold in *Pthrp* null fetuses compared with wild-type (WT) fetuses and may have prevented more severe hypocalcemia.[5] However, the biochemical abnormalities in *Pthrp* null fetuses indicate either that PTH could not fully compensate for the absence of PTHrP or that it was restrained by the actions of the parathyroid calcium-sensing receptor (CaSR) to maintain the lower, adult-normal value of serum calcium.[6] These observations reaffirmed the hypothesis that PTHrP is an important regulator of fetal mineral homeostasis, but further study of the role of fetal PTH was warranted.

We next examined *Hoxa3* null fetuses as a model for aparathyroidism; these mice also lack the thymus and are completely devoid of PTH.[7] We found more profound hypocalcemia than in *Pthrp* null fetuses such that the blood calcium level

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was reduced well below the ambient maternal calcium concentration.[5,8] *Hoxa3* nulls also had hypomagnesemia, hyperphosphatemia, a normal rate of placental calcium transfer, low amniotic fluid mineral content, and reduced skeletal calcium and magnesium content. We also created and studied *Hoxa3/Pthrp* double mutants that lacked both PTH and PTHrP. These double-mutant fetuses had much lower blood calcium and skeletal mineral content than either of the single mutants; their phenotype was similar to what we observed in mice lacking the PTH/PTHrP receptor.[4,5]

Thus our previous investigations showed that absence of parathyroids caused a more substantial reduction in serum calcium and skeletal mineral content than absence of PTHrP despite the fact that PTH normally circulates at low levels in the fetus. Removing both parathyroids and PTHrP (or the PTH/PTHrP receptor) caused even more severe abnormalities, suggesting that PTH can partly compensate for the absence of PTHrP or that both PTH and PTHrP normally contribute to the regulation of fetal blood calcium and skeletal mineralization. However, *Hoxa3* null mice have other abnormalities in tissues derived from the third pharyngeal arch that contribute to their mortality after birth and which may affect their biochemical and skeletal phenotype. Thus the specific role of PTH in fetal calcium homeostasis required further investigation.

In this study, we hypothesized that despite its low circulating levels, PTH plays a critical role in regulating fetal calcium homeostasis and skeletal mineralization. To address this—and to avoid being led astray by possibly confounding features of one mutant model—we examined *two* different genetic models of PTH deficiency. *Pth* null mice have enlarged parathyroids that are incapable of making PTH; the adults display hypocalcemia and hyperphosphatemia.[9] *Gcm2* null mice lack parathyroids but have PTH that arises from the thymus; the adults also have hypocalcemia and hyperphosphatemia.[10] *Pth* nulls served as a model of complete absence of PTH, whereas *Gcm2* nulls were a model of severe hypoparathyroidism. In both colonies, the pups are born in the expected Mendelian ratios, but survival of null mice is reduced after birth. We have observed that this is due, in part, to the mother, who selectively tosses these otherwise healthy-appearing pups from the nest (even if the investigator puts them back in); there also may be postnatal hypocalcemia-related deaths. In both colonies, the null mice that survive are fertile and grossly indistinguishable from their WT and heterozygous littermates.

Materials and Methods

Animal husbandry

The creation of *Pth* null and *Gcm2* null mice has been described previously.[9,10] The original strains were back-crossed into Black Swiss (Taconic, Germantown, NY, USA) for three generations prior to beginning any studies and at least annually thereafter such that the mice are about 10 generations into Black Swiss. The colonies were maintained by breeding heterozygous-deleted mice together. Genotyping was done by PCR on DNA extracted from tail clips of weaned pups. Mice were mated overnight; the presence of a vaginal mucus plug on the morning after mating marked embryonic day (ED) 0.5. Normal gestation in these mice is 19 days. All mice were given a standard chow (1% calcium, 0.75% phosphorus) diet and water ad libitum. All studies were performed with the prior approval of the Institutional Animal Care Committee of Memorial University of Newfoundland.

Chemical and hormone assays

Whole blood, plasma, serum, and amniotic fluid were collected using methods described previously.[8] Ionized calcium was measured on whole blood using a Chiron Diagnostics 634 Ca^{2+}/pH Analyzer (Chiron Diagnostics, East Walpole, MA). Total calcium, phosphate, and magnesium were measured using colorimetric assays in the *Gcm2* colony (Sigma-Aldrich, Oakville, Ontario, Canada); discontinuation of these kits necessitated that different colorimetric assays had to be used in the *Pth* colony (Diagnostic Chemicals Limited, Charlottetown, Prince Edward Island, Canada). PTH was measured with a rodent PTH 1-34 Elisa kit that has a detection limit of 1.6 pg/mL (Immutopics, San Clemente, CA, USA). Plasma PTHrP was measured using a sensitive RIA with an antibody directed to an amino-terminal epitope on samples that had been collected in a cocktail of aprotinin and EDTA.[11] WT fetuses typically have values of 6 to 8 pmol/L, whereas *Pthrp* null fetuses had values of 3.8 ± 0.3 pmol/L; this likely represents the detection limit of the assay in fetal mouse plasma[8] (and unpublished data).

Placental calcium transfer

This procedure has been described in detail elsewhere.[4] Briefly, pregnant dams on ED 18.5 were given an intracardiac injection of 50 μCi ^{45}Ca and 50 μCi of ^{51}Cr-EDTA. Five minutes later, the dams were sacrificed, and each fetus was removed from its placenta. The ratio of ^{45}Ca to ^{51}Cr radioactivity within each fetus was measured using a gamma counter and a liquid scintillation counter, respectively. The mean $^{45}Ca/^{51}Cr$ activity ratio of the heterozygous fetuses in each litter was set at 100% in order that the results from different litters could be pooled for analysis.

Fetal PTH treatment

On ED 18.5, we exposed the uteri of *Pth* heterozygous dams and gave half the fetuses an intraabdominal injection of 1 nmol rat PTH 1-84 (Bachem, Torrance, CA) in 2 μL saline, whereas the remaining fetuses received 2 μL saline. Sutures were placed over the gestational sacs of the PTH-injected fetuses so that they could be identified later. The mother's incision was closed, and she was permitted to awaken and move about normally. Eighty-five minutes after the fetal injections, the placental calcium transfer procedure described earlier was carried out (ending 90 minutes from the time of PTH or saline injection). In separate experiments (no radioactivity) to determine the effect of PTH treatment on placental gene expression, at 90 minutes after the fetal injections, the placentas were removed, snap frozen with liquid nitrogen, and stored at −70°C for subsequent RNA extraction, microarray, and real-time quantitative RT-PCR.

Fetal ash and skeletal mineral assay

As described previously,[5] intact fetuses (ED 18.5) were reduced to ash in a furnace (500°C × 24 hours). A Perkin Elmer 2380

Atomic Absorption Flame Spectrophotometer (Norwalk, CT) determined the calcium and magnesium content of the ash.

Alizarin red S and alcian blue preparations

As described in detail previously,[5] fresh fetuses were fixed in 95% EtOH followed by clearing with acetone, stained with alcian blue 8GS and alizarin red S, and then immersed in 1% aqueous KOH until the fetal skeleton was clearly visible through the surrounding tissue. They were transferred into 100% glycerine for permanent storage.

Histology

Undecalcified fetal tibiae were fixed in paraformaldehyde, dehydrated in graded alcohol series, and embedded in paraffin. Then 5 μm sections were deparaffinized, rehydrated, and transferred to distilled water. For von Kossa staining, the sections were transferred to 1% aqueous silver nitrate solution and exposed for 45 minutes under a strong light. They then were washed thrice in distilled water, placed in 2.5% sodium thiosulfate (5 minutes), and washed thrice again in distilled water. Finally, they were counterstained with methyl green, dehydrated in 1-butanol and xylene, and mounted.

RNA extraction

RNA was extracted from snap-frozen placentas and anterior neck sections using an RNeasy Midi Kit (Qiagen, Valencia, CA). The placental samples were representative of the entire placenta and included the three trophoblast cell types and intraplacental yolk sac; the maternal contribution is limited to endothelial cells that line maternal blood vessels. Quality of the RNA samples used for microarray analysis was assessed with the Agilent 2100 BioAnalyzer (Agilent Technologies, Palo Alto, CA), whereas the quality of other RNA samples was assessed using ultraviolet (UV) spectrophotometry and inspection of ribosomal RNA integrity on the electrophoresced gel.

Microarray

Placental RNA from PTH- or saline-treated fetuses was analyzed at the Centre for Applied Genomics, Microarray Facility, Hospital for Sick Children (Toronto, Ontario, Canada). The Mouse Gene ST 1.0 Array (Affymetrix, Santa Clara, CA) was completed on 12 samples (one sample per chip), representing three samples for each of the four groups: WT and *Pth* null, each treated with either saline or PTH (1-84).

Primary data analysis at the Statistical Analysis Core Facility of the Centre for Applied Genomics used the March 2008 gene annotation information from Affymetrix. Probesets without gene names/gene assignments were removed, leaving 22,158 probesets. Raw data were normalized using the robust multiarray average (RMA) algorithm,[12] and differentially expressed genes then were identified using the local-pooled-error test (LPE).[13] False discovery rate (FDR)[14] was set at 0.05 such that genes with adjusted p values of less than .05 were considered to be statistically significant. The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE16983 (www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE16983).

Real-time quantitative RT-PCR

We used TaqMan Gene Expression Assays, which are predesigned primers and probes for optimal amplification, to determine expression of S100g (CaBP-9K), VDR, PTHrP, PTH, and TRPV6. In addition to the TaqMan Gene Expression Assays, TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA) was used when PTHrP expression was assessed in both neck and placental RNA samples. Details of conditions and cycle times have been reported previously.[15] To assess S100g (CaBP-9K), VDR, PTH, and TRPV6 expression in placental samples, we used the TaqMan RNA-to-C_T 1-Step Kit (Applied Biosystems) in addition to the TaqMan Gene Expression Assays. This eliminated the need to carry out a separate cDNA synthesis step prior to real-time quantitative RT-PCR. With use of the TaqMan RNA-to-C_T 1-Step Kit, the thermal cycler protocol then consisted of a 15 minute cycle at 48°C, a 10 minute cycle at 95°C, followed by 40 cycles of 15 seconds at 95 °C and 1 minute at 60°C. We performed all real-time quantitative RT-PCR using the ABI PRISM 7000 Sequence Detection System (Applied Biosystems), as described previously.[16] All samples were analyzed in triplicate. Relative expression ratios were representative of the threshold cycle (the PCR cycle at which an increase in reporter fluorescence is above a baseline signal) normalized to GAPDH and compared with WT animals.

Statistical analysis

Data were analyzed using SYSTAT 5.2.1 for Macintosh (SYSTAT, Inc., Evanston, IL, USA). ANOVA was used for the initial analysis; a post hoc test was used to determine which pairs of means differed significantly from each other. Real-time PCR results were analyzed by the $2^{-\Delta\Delta CT}$ method, where the target and reference are amplified in separate wells.[17] Two-tailed probabilities are reported, and all data are presented as mean ± SE.

Results

Serum chemistries

As expected, serum PTH was undetectable in all *Pth* null fetuses (Fig. 1*A*). In *Gcm2* null fetuses, the PTH level ranged from low to undetectable, indicating that some fetuses had circulating PTH likely arising from the thymus (see Fig. 1*B*). Both *Pth* null and *Gcm2* null fetuses shared identical phenotypes of hypocalcemia, hypomagnesemia, and hyperphosphatemia (Table 1); this pattern is very similar to the phenotype of *Pthrp* null fetuses in the same genetic background. The ionized calcium was reduced to the maternal level, equal to the level of *Pthrp* null fetuses but well above the level observed in aparathyroid *Hoxa3* null fetuses within the same genetic background (see Fig. 1*C*, *D*). Amniotic fluid calcium and magnesium were low in both *Pth* null and *Gcm2* null fetuses (see Table 1), which is consistent with reduced renal filtered load from the lower serum levels of these minerals.

Response of PTHrP

We had observed previously that PTH upregulates in the absence of PTHrP (i.e., in *Pthrp* null fetuses); we now examined whether PTHrP upregulates in response to hypocalcemia and absence of PTH. Circulating plasma PTHrP levels were no different from respective WT and heterozygous littermates (see Fig. 1*E*, *F*). The

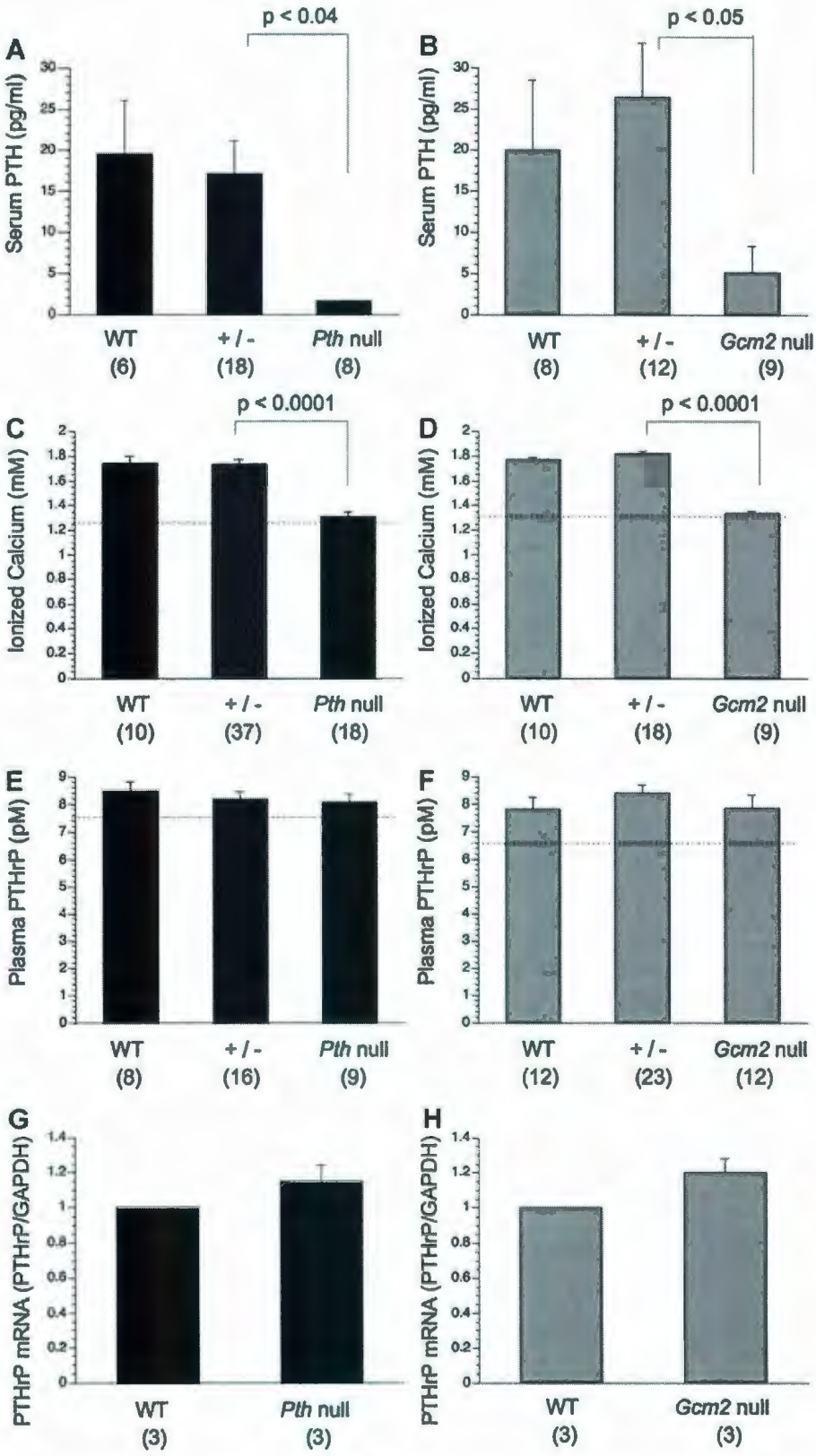

Fig. 1. Lack of PTH causes hypocalcemia but no compensatory increase in PTHrP in either *Pth* null or *Gcm2* null fetuses. Serum PTH was undetectable (< 1.6 pg/mL) in all *Pth* nulls (*A*) and low but detectable (5.0 pg/mL) in *Gcm2* nulls (*B*). Both null genotypes had hypocalcemia (equal to the maternal calcium concentration) (*C, D*) but no alteration in plasma PTHrP (*E, F*) and no upregulation of PTHrP mRNA (as detected by real-time quantitative RT-PCR) in the neck region that contains the parathyroids (*G, H*). Fetuses from the *Pth* colony are shown in black; those from the *Gcm2* colony are shown in gray. Numbers in parentheses indicate the numbers of pups studied. Dashed lines indicate the mean maternal concentrations for each parameter, which were collected and measured simultaneously with the fetal samples.

Table 1. Biochemical Weight and Skeletal Mineral Measurements from *Pth* and *Gcm2* Null Fetuses Versus Respective Siblings and Mothers

	Pth Colony				*Gcm2* Colony			
	WT	±	Null	Maternal	WT	±	Null	Maternal
Ca^{2+} (mM)	1.75 ± 0.05	1.74 ± 0.03	1.31 ± 0.04*	1.21 ± 0.06	1.77 ± 0.02	1.82 ± 0.02	1.34 ± 0.03*	1.31 ± 0.03
Mg (mM)	1.17 ± 0.13	1.32 ± 0.07	1.11 ± 0.10***	0.80 ± 0.09	0.90 ± 0.01	0.92 ± 0.01	0.87 ± 0.01**	0.88 ± 0.02
PO_4 (mM)	2.97 ± 0.17	3.03 ± 0.11	3.54 ± 0.11**	2.45 ± 0.20	3.23 ± 0.26	3.32 ± 0.16	4.57 ± 0.15*	3.18 ± 0.17
Amniotic total Ca (Mm)	2.19 ± 0.14	2.14 ± 0.10	1.50 ± 0.15**	—	2.35 ± 0.17	2.23 ± 0.10	1.75 ± 0.13**	—
Amniotic Mg (mM)	1.44 ± 0.09	1.35 ± 0.07	0.88 ± 0.12**	—	1.15 ± 0.07	1.20 ± 0.04	0.90 ± 0.07**	—
Weight (g)	1.03 ± 0.03	1.02 ± 0.02	1.03 ± 0.03	—	1.03 ± 0.03	1.05 ± 0.02	1.03 ± 0.02	—
Ash (mg)	19.1 ± 0.5	19.7 ± 0.4	18.1 ± 0.5*	—	20.1 ± 0.5	21.1 ± 0.3	18.0 ± 0.4*	—
Calcium (mg/g ash)	69.5 ± 2.5	69.4 ± 1.7	59.6 ± 2.3*	—	58.9 ± 2.2	64.9 ± 1.2	47.8 ± 1.6*	—
Magnesium (mg/g ash)	24.3 ± 1.0	22.4 ± 0.7	19.8 ± 0.9*	—	21.0 ± 0.7	20.5 ± 0.4	18.5 ± 0.5*	—

Note that the kits used to measure total Ca, Mg, and PO_4 in the *Pth* colony were not the same as the kits used for the *Gcm2* colony because the manufacturer discontinued the original kits; thus the results for total Ca, Mg, and PO_4 are not directly comparable between the two colonies.

*$p < .001$; **$p < .01$; ***$p < .08$ versus respective heterozygous siblings.

lack of a difference is likely real because this assay previously distinguished *Pthrp* null fetuses from WT fetuses and also found very high (63.5 pmol/L) PTHrP levels in PTH/PTHrP receptor (*Pthr1*) null fetuses.[8] However, in order to rule out a local increase in PTHrP within fetal parathyroids, we extracted RNA from anterior neck sections that included the parathyroids and found no elevation in PTHrP mRNA by quantitative real-time RT-PCR (see Fig. 1*G*, *H*).

Skeletal phenotype

Both *Pth* null and *Gcm2* null fetuses showed a grossly normal skeletal phenotype, as shown by alizarin red S– and alcian blue–stained intact specimens in Fig. 2(*A*, *B*). In an earlier report, the tibial diaphysis was significantly shortened in *Pth* nulls from an inbred C57BL/6 background,[9] but we observed that long bone and tibial diaphyseal lengths of both *Pth* null and *Gcm2* null

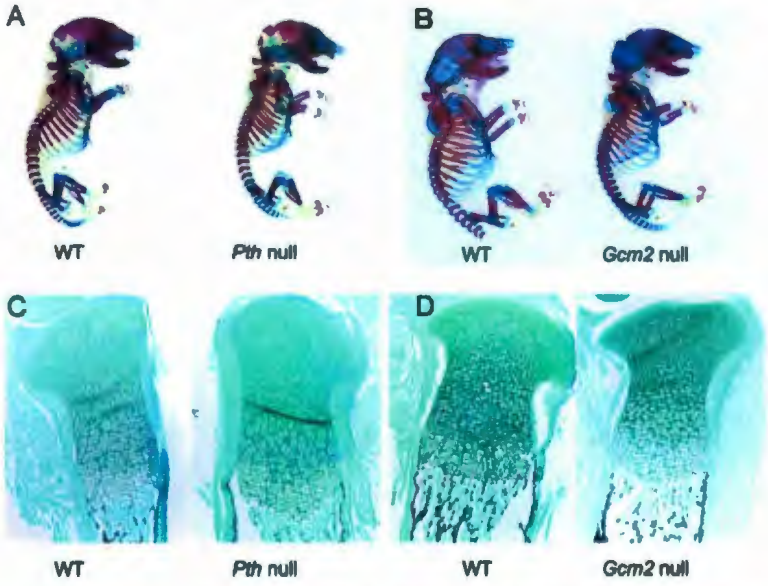

Fig. 2. Gross and microscopic skeletal morphology of ED 18.5 WT, *Pth* null, and *Gcm2* null fetuses. Skeletal preparations stained with alizarin red S (for mineral) and alcian blue (for cartilage) show that both *Pth* null fetuses (*A*) and *Gcm2* null fetuses (*B*) had normal axial and appendicular skeletons, including lengths of long bones and mineralization pattern. Panels *C* and *D* display von Kossa–stained tibial sections that were counterstained with methyl green. *Pth* null and *Gcm2* null tibiae showed apparently normal endochondral development with no alteration in the lengths or cellular morphology of the cartilaginous or bony compartments, although less mineral (detected by von Kossa) appeared to be present in both null genotypes.

fetuses (in an outbred Black Swiss background) were normal at gross and microscopic levels (see Fig. 2*A–D*). Body weights of the *Pth* null and *Gcm2* null fetuses were no different from those of their respective WT siblings (see Table 1). Histologic sections demonstrated normal endochondral bone development with no alteration in the length or morphology of the cartilaginous zones and the growth plate (see Fig. 2*C, D*). Von Kossa staining suggested a modest reduction in skeletal mineral content that was confirmed by ash weight and mineral content measurements in both phenotypes (see Fig. 2*C, D* and Table 1). These reductions in skeletal calcium and magnesium content were about half of what we had observed previously in *Hoxa3* null fetuses.[5]

Placental calcium transfer

Fetal hypocalcemia might be caused by a reduction in maternal-fetal calcium flux; conversely, fetal hypocalcemia might induce a compensatory increase in maternal-fetal calcium transport. However, in *Pth* null fetuses, no alteration in placental ^{45}Ca transfer was noted (Fig. 3*A*). In *Gcm2* null fetuses, a modest but statistically significant increase in ^{45}Ca transfer was detected (see Fig. 3*B*). To test whether PTHrP might explain this increase in placental calcium transfer, we assayed placental PTHrP mRNA by quantitative real-time RT-PCR and found no increase in PTHrP in either *Pth* null or *Gcm2* null fetuses (see Fig. 3*C, D*). As noted earlier, circulating PTHrP levels were not increased in *Gcm2* nulls either.

Role of PTH in placental calcium transfer

Whether PTH contributes to the regulation of placental calcium transfer has been unclear, whereas midmolecular fragments of PTHrP have been shown to stimulate this process in fetal lambs and mice.[4,18,19] Our previous studies in *Pthrp* null fetuses had shown no effect of PTH treatment, but those fetuses already had threefold upregulation of endogenous PTH and therefore might have been unable to respond to administration of exogenous PTH.[4,5] We used *Pth* null fetuses as a model to test the ability of PTH treatment to increase the rate of maternal-fetal calcium transfer. We treated all fetuses in utero with injections of either saline or a dose of PTH 1-84 that was equimolar to the dose of PTHrP 1-86 that had proved effective in *Pthrp* null fetuses. We then assayed placental calcium transfer 90 minutes after

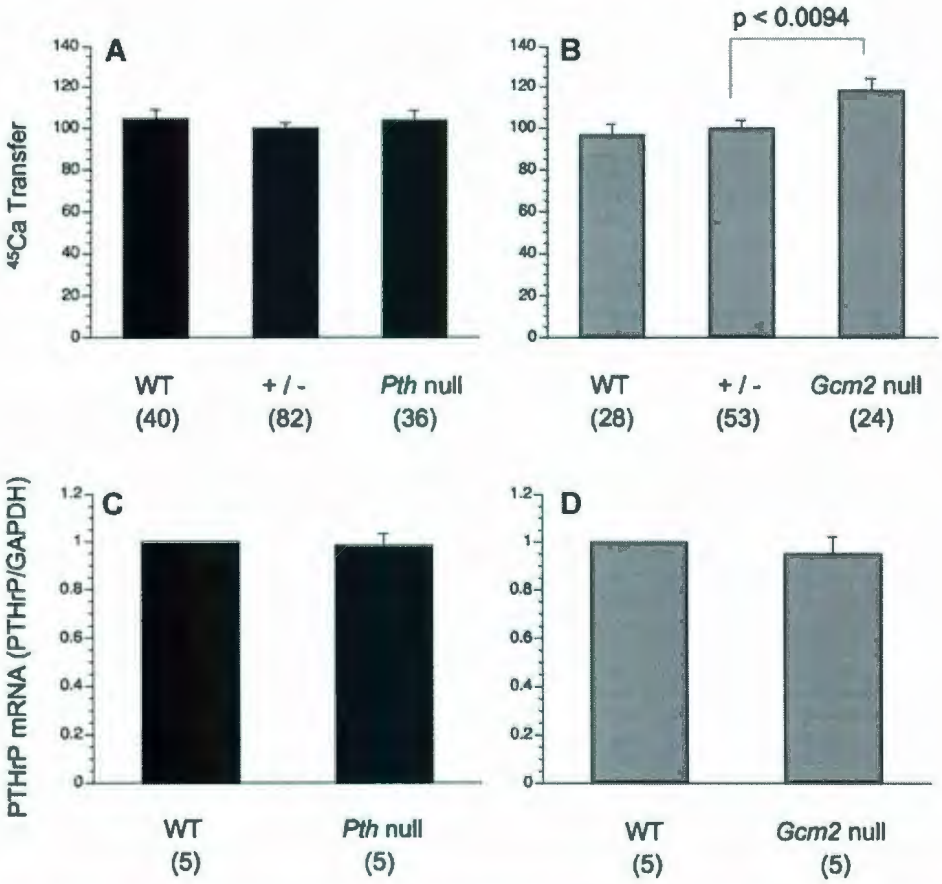

Fig. 3. Placental calcium transfer and placental PTHrP mRNA. After administration of ^{45}Ca and ^{51}Cr-EDTA to the mother, *Pth* null fetuses showed no alteration in the relative transfer of ^{45}Ca compared with WT and *Pth*$^{+/-}$ littermates (*A*). Conversely, *Gcm2* null fetuses showed a significant upregulation in ^{45}Ca accumulation (*B*). This increase in placental calcium transfer was not due to a compensatory increase in PTHrP. Placental PTHrP mRNA was unaltered in *Pth* null (*C*) and *Gcm2* null (*D*) fetuses, as assessed by real-time quantitative RT-PCR. Figure 1(*E, F*) showed that plasma PTHrP also was unaltered in either genotype. Fetuses from the *Pth* colony are shown in black; those from the *Gcm2* colony are shown in gray. Numbers in parentheses indicate the numbers of pups studied.

treatment. A statistically significant increase in placental calcium transfer occurred in *Pth* null fetuses (Fig. 4). This increase in ^{45}Ca accumulation was not secondary to altered systemic calcium homeostasis because PTH treatment did not alter the fetal ionized calcium (1.29 ± 0.09 mM in PTH-treated versus 1.31 ± 0.10 mM in saline-treated *Pth* null fetuses, $p =$ NS).

To identify possible mechanisms through which PTH might be acting on the placenta, we extracted RNA from placentas of PTH- or saline-injected WT and *Pth* null fetuses and performed a genome-wide microarray. At baseline, *Pth* null placentas had a 60% reduction in TRPV6 mRNA, a 40% reduction in calbindin D-9K mRNA, and a 20% reduction in VDR mRNA versus WT placentas; the expression of other genes involved in cation and solute transport also was significantly reduced (Table 2). The expression of TRPV6, calbindin D-9K, and VDR mRNAs was assessed independently by quantitative real-time RT-PCR, and each was reduced by 60% to 80% in the *Pth* null versus WT placentas (Fig. 5*A–C*). In response to PTH administration, *Pth* null placentas had a 1.8-fold increase in VDR mRNA, and the expression of several solute carriers also increased (Table 3). Quantitative real-time RT-PCR confirmed that the VDR mRNA had increased 1.5-fold over baseline in response to PTH 1-84 treatment (see Fig. 5*D*).

Comparison of *Pth* and *Gcm2* null placentas and detection of placental PTH

We examined the expression TRPV6, calbindin D-9K, and VDR mRNAs in *Gcm2* null placentas and found that TRPV6 and calbindin D-9K were reduced by 80% and 55%, respectively, compared with their WT siblings ($p < .001$), whereas VDR mRNA was unchanged from the WT value ($p =$ NS; data not shown). The real-time RT-PCR then was repeated using RNA from *Gcm2* and *Pth* null placentas compared side by side. The relative expressions of TRPV6, calbindin D-9K, and VDR all were significantly higher in *Gcm2* null placentas than in *Pth* nulls (Fig. 6*A–C*).

Our findings prompted the consideration that PTH might be expressed in the placenta; if so, it should be absent in *Pth* nulls but present in *Gcm2* nulls. By real-time quantitative RT-PCR, PTH mRNA was detected in WT placentas obtained from both colonies; furthermore, PTH expression was 1.2-fold higher in *Gcm2* null placentas than in WT placentas ($p =$ NS). Direct comparison of RNA from *Gcm2* and *Pth* null placentas studied side by side by real-time PCR showed PTH expression to be almost sevenfold higher in *Gcm2* null placentas than in *Pth* null placentas ($p < .001$) (see Fig. 6*D*). Expression in *Pth* nulls did not appear until the thirty-first cycle and may indicate a false-positive detection or the presence of maternal sources of PTH mRNA.

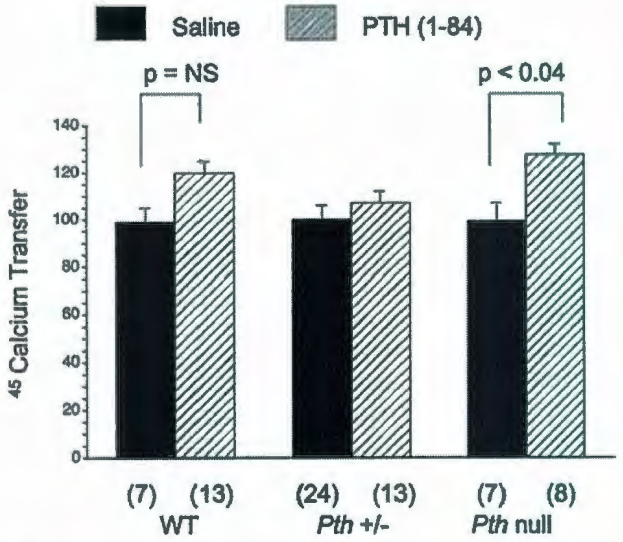

Fig. 4. PTH 1-84 stimulates placental calcium transfer in *Pth* null fetuses. Treatment in utero with 1 nmol PTH 1-84 versus saline resulted in a significant increase in the transfer of ^{45}Ca to *Pth* null fetuses. Saline-injected fetuses are shown in black bars; PTH 1-84–injected fetuses are shown in hatched bars. Numbers in parentheses indicate the numbers of pups studied.

Discussion

The PTH/PTHrP receptor is well known to mediate many of the N-terminal actions of PTH and PTHrP. An unresolved paradox is how these two ligands can both be present in the fetal circulation but still carry out distinct roles. We undertook the current studies to further clarify the role of PTH, expecting to see a phenotype identical to aparathyroid *Hoxa3* null fetuses—that is, marked hypocalcemia (well below the maternal calcium level), hypomagnesemia, hyperphosphatemia, low amniotic fluid mineral content, and a 25% reduction in skeletal calcium content. Instead, we found a milder hypoparathyroid phenotype with modestly reduced blood calcium concentration (equal to the maternal level), hyperphosphatemia, low amniotic fluid mineral content, and a 10% reduction in skeletal calcium content.

The modestly reduced skeletal mineral content of *Pth* null and *Gcm2* null fetuses confirms that PTH is required to achieve normal mineralization of the skeleton prior to term. The role of PTH may be to directly drive skeletal mineralization by stimulating osteoblast function or to maintain the normally high fetal blood calcium concentration and thereby facilitate mineralization of newly formed bone. Absence of PTH did not alter endochondral bone development or limb lengths in either genetic model, which suggests that the role of PTH to facilitate mineralization may be through its role to maintain the serum calcium concentration and not through any effect on chondrocyte or osteoblast physiology. The original report of *Pth* null fetuses had indicated significant shortening of the tibial diaphysis, whereas we observed no such shortening in *Pth* or *Gcm2* null fetuses[9]; the difference between that report and this one may be the respective genetic backgrounds of the mice (C57BL/6 versus Black Swiss). The fact that two distinct models of PTH deficiency shared the same skeletal phenotype suggests that it was caused directly by PTH deficiency and not confounded by unknown factors.

In our prior studies, the phenotype of lower blood calcium and reduced skeletal mineral content was progressively more severe in aparathyroid *Hoxa3* fetuses and PTH/PTHrP receptor (*Pthr1*) null fetuses.[5] In this study, two different models of PTH deficiency in animals of the same genetic background had

Table 2. Selected List of Genes Showing Statistically Significant Differential Regulation at Baseline (i.e., Saline Injection) Between *Pth* Null Versus WT Placentas

Gene	Gene name	Fold change	Adjusted *p* value
Fabp1	Fatty acid binding protein 1	2.11	.013
Hbb-y	Hemoglobin Y, beta-like embryonic chain	1.88	.001
Krt1	Keratin 1	1.54	.049
Atp7b	ATPase, Cu^{2+} transporting, beta-polypeptide	0.96	.014
Osbpl6	Oxysterol binding protein-like 6	0.84	.002
Afp	Alpha-fetoprotein	0.84	<.001
Lrp2 (megalin)	Low-density lipoprotein receptor–related protein 2	0.83	<<.001
Dab2	Disabled homologue 2 (*Drosophila*)	0.82	<<.001
8430408G22Rik	RIKEN cDNA 8430408G22 gene	0.82	.044
Slc27a2	Solute carrier family 27 (fatty acid transporter)	0.81	<<.001
Ahsg	α-2-HS-glycoprotein	0.81	<.001
Vdr	Vitamin D receptor	0.81	<<.001
Apoc1	Apolipoprotein C-I	0.80	.019
Heph	Hephaestin	0.80	<<.001
Abcc2	ATP-binding cassette	0.79	<.001
Slc13a3	Solute carrier family 13 (sodium-dependent dicarboxylate transporter)	0.76	.016
Slc7a9	Solute carrier family 7 (cationic amino acid transporter	0.76	<<.001
Apoa2	Apolipoprotein A-II	0.76	<<.001
Cubn	Cubilin (intrinsic factor-cobalamin receptor)	0.75	<<.001
2010003K11Rik	RIKEN cDNA 2010003K11 gene	0.75	.002
Apom	Apolipoprotein M	0.75	<<.001
Trf	Transferrin	0.75	<<.001
5033414D02Rik	RIKEN cDNA 5033414D02 gene	0.74	<.001
Slc22a2	Solute carrier family 22 (organic cation transporter)	0.73	.001
Apoc2	Apolipoprotein C-II	0.72	<<.001
Apob	Apolipoprotein B	0.72	<<.001
Rbp2	Retinol-binding protein 2	0.71	<<.001
Ambp	α_1-Microglobulin	0.70	<.001
Slc6a19	Solute carrier family 6 (neurotransmitter transporter)	0.69	<.001
Slc5a1	Solute carrier family 5 (sodium/glucose cotransporter)	0.69	<<.001
Apoa1	Apolipoprotein A-I	0.68	<<.001
Gc (*DBP*)	Group specific component (vitamin D–binding protein)	0.67	<<.001
1300017J02Rik	RIKEN cDNA 1300017J02 gene	0.66	<<.001
Ttr	Transthyretin	0.66	<<.001
Sfpd	Surfactant-associated protein D	0.65	.003
Slc3a1	Solute carrier family 3	0.64	<<.001
Apoa4	Apolipoprotein A-IV	0.64	<<.001
Alb	Albumin	0.63	<<.001
Mcoln3	Mucolipin 3	0.62	.041
S100g (*CaBP-D9K*)	S100 calcium–binding protein G (calbindin D9k)	0.58	<<.001
Aqp8	Aquaporin 8	0.46	<<.001
Trpv6	Transient receptor potential cation channel, subfamily V, member 6	0.40	<<.001

modestly reduced blood calcium and skeletal mineral content. The more modest phenotype of the PTH-deficiency models may indicate that parathyroid tissue participates in regulating fetal mineral homeostasis through the release of other as yet unidentified factors. Further comparative study of other parathyroid-deleted mutants (*Pax1* and others) and double mutants of *Pth* and *Hoxa3* may reveal why genetic deletion of parathyroids in *Hoxa3* nulls or absence of the PTH/PTHrP receptor in *Pthr1* nulls caused more severe hypocalcemia and skeletal undermineralization than in *Pth* null and *Gcm2* null fetuses.

In both *Pth* null and *Gcm2* null fetuses there was no increase in PTHrP mRNA expression in the neck or placenta, no increase in plasma PTHrP, and no reduction in placental calcium transfer.

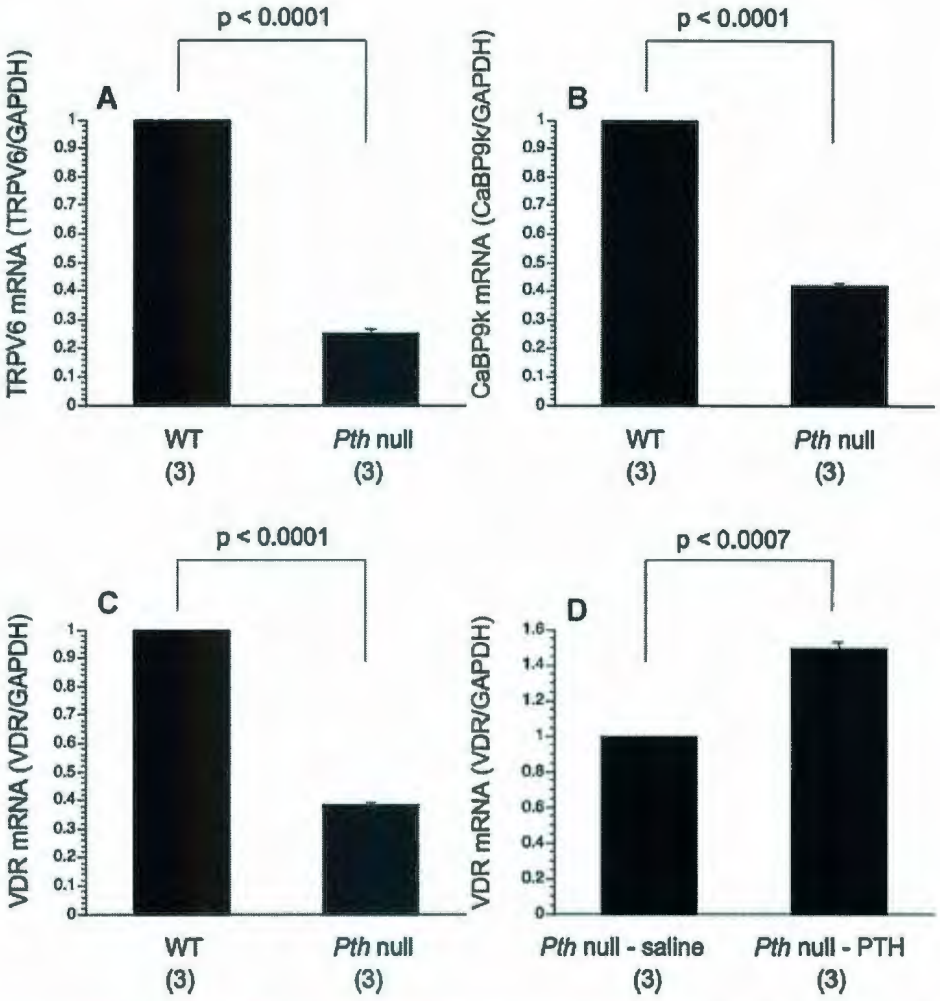

Fig. 5. Placental expression of TRPV6, calbindin D-9k, and VDR. Placentas were harvested from PTH 1-84– and saline-treated fetuses and analyzed by genome-wide microarray. Saline-treated *Pth* null placentas showed significant downregulation versus WT placentas in the expression of mRNAs for TRPV6 (*A*), calbindin D-9k (*B*), and VDR (*C*). Conversely, following PTH 1-84 treatment, *Pth* null placentas showed significant upregulation of VDR mRNA versus saline-treated *Pth* null placentas (*D*). Numbers in parentheses indicate the number of placentas studied.

Table 3. Selected List of Genes Showing Differential Regulation at 90 Minutes Between PTH 1-84–Injected and Saline-Injected *Pth* Null Placentas

Gene	Gene name	Fold change	Adjusted *p* value
2010109I03Rik	RIKEN cDNA 2010109I03 gene	3.68	<<.001
Slc39a8	Solute carrier family 39 (metal ion transporter)	2.20	<<.001
Sfpd	Surfactant-associated protein D	1.90	.067
Vdr	Vitamin D receptor	1.79	<<.001
Alb	Albumin (Alb)	1.78	<.001
2210415F13Rik	RIKEN cDNA 2210415F13 gene	1.55	<.001
Slc23a3	Solute carrier family 23 (nucleobase transporters)	1.43	.083
Gc (DBP)	Group-specific component (vitamin D–binding protein)	1.40	<<.001
Apoa4	Apolipoprotein A-IV	1.26	<<.001
1300017J02Rik	RIKEN cDNA 1300017J02 gene	1.25	.012
Rbp2	Retinol-binding protein 2	1.20	<.001
Cubn	Cubilin (intrinsic factor-cobalamin receptor)	0.89	.001

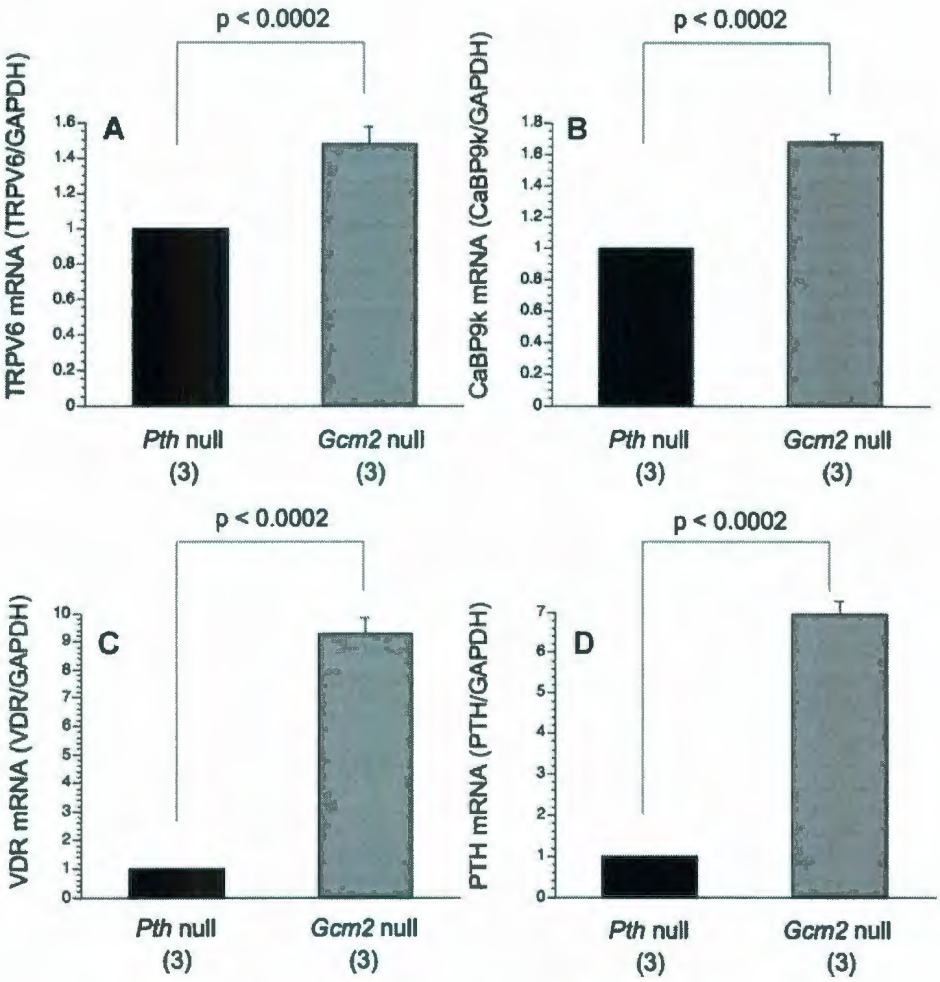

Fig. 6. Placental expression of TRPV6, calbindin D-9k, VDR, and PTH in *Gcm2* versus *Pth* null placentas. Compared with their respective WT counterparts, *Gcm2* nulls had reduced expression of TRPV6 and calbindin D-9k but normal expression of VDR (not shown). When compared simultaneously with *Pth* null placentas, *Gcm2* null placentas had significantly higher expression of TRPV6 (*A*), calbindin D-9k (*B*), and VDR (*C*). WT and *Gcm2* null placentas expressed PTH (not shown), and direct comparison of *Gcm2* null with *Pth* null placentas revealed a sevenfold higher expression of PTH mRNA in *Gcm2* null placentas (*D*). Numbers in parentheses indicate the numbers of placentas studied.

Similarly, we reported earlier that despite more profound hypocalcemia, *Hoxa3* null fetuses had no upregulation of placental PTHrP mRNA, no increase in plasma PTHrP, and no alteration in placental calcium transfer. These findings indicate that PTHrP must be regulated differently from PTH during fetal life. PTH is regulated by the CaSR on parathyroids, increasing in response to fetal hypocalcemia (such as in *Pthrp* null fetuses[5] and maternal hypocalcemia[20]) and increasing in response to inactivating mutations of the CaSR.[6] In contrast, fetal PTHrP does not respond to any of these stimuli; the only situation in which we have found PTHrP to be increased is in *Pthr1* null fetuses, which lack the PTH/PTHrP receptor.[8] PTHrP may be produced autonomously by the placenta or regulated by other factors, such as the sensing of the calcium content exchanged across the trophoblasts and intraplacental yolk sac.[3,21]

We observed that *Pth* null placentas had reduced mRNAs for TRPV6, calbindin D-9K, VDR, vitamin D–binding protein, and other solute or cation transporters. The independent effect of some of these alterations can be predicted from our previous studies. First, in collaboration with the Hediger Laboratory, we found that ablation of TRPV6 in mice significantly lowered placental calcium transfer.[22] Second, we noted reduced expression of calbindin D-9K in the intraplacental yolk sac in *Pthrp* nulls, which have reduced placental calcium transfer.[4,21] Third, ablation of VDR (*Vdr* null fetuses) was associated with normocalcemia but an *increased* rate of placental calcium transfer and increased placental expression of TRPV6.[16] The studies in *Vdr* null fetuses may indicate that calcitriol and the VDR act as a brake on the rate of placental calcium transfer; in the absence of VDR, TRPV6 expression and placental calcium transfer increase. Taken together, these previous studies predict that some of the altered gene expression in *Pth* null placentas should increase net placental calcium transfer, whereas other alterations should decrease it. The decreased expression of TRPV6, calbindin D-9K, and VDR (and other factors) may have offset each other to lead to no net change in placental calcium transfer, exactly as we observed in *Pth* null fetuses.

Although placental calcium transfer was not reduced in the absence of PTH in either *Pth* null or *Gcm2* null fetuses, the blood calcium, amniotic fluid calcium, and skeletal mineral

content all were reduced. This suggests that the rate of backflux or reverse flow of calcium from fetus to mother must have been increased in order to account for where the mineral went. The short 5 minute interval between administration of the isotopes and removal of each fetus from its placenta in this technique means that largely forward flow from mother to fetus is measured, whereas backflux is not. Within this time frame, the transferred isotopes become diluted in the total blood volume of each fetus; consequently, only a small amount of isotope can be expected to return via the umbilical artery and across the placenta to the mother within 5 minutes. The effect of backflux became apparent in our previous studies of *Pthrp* knockout mice, where we observed that the relative difference in placental calcium transfer among WT, *Pthrp*$^{+/-}$, and *Pthrp* null fetuses increased from 5 to 15 to 30 minutes, likely indicating the progressive effect of backflux on the observed net fetal accumulation of isotopes. Consequently, we have since used the 5 minute time point in the placental calcium transfer experiment because this is unlikely to be confounded by backflux of isotope.

The placental calcium-transfer methodology used in our studies has the advantage of studying intact fetuses with very minimal intervention: an intracardiac injection of isotopes given to a pregnant mouse that is anesthetized for less than 30 seconds. Typically, fetuses from 6 to 10 pregnant mice are required to compare the baseline rate of placental calcium transfer among WT, heterozygous, and null fetuses. The number of mice required more than doubles in order to compare the effect of a single active treatment versus saline at one time point, and measurements can be done only at a single time point after injection (it is a terminal experiment). Consequently, it was not feasible to study a time course of PTH injections or determine a full dose-response curve. Thus we chose the dose of PTH and time point based on our earlier studies in which an equimolar dose of PTHrP 1-86 or 67-86 increased placental calcium transfer in *Pthrp* null fetuses.[4] We demonstrated an increase in placental calcium transfer with PTH treatment of *Pth* null fetuses, which suggests that PTH may contribute to the normal regulation of placental calcium transfer. Our finding is consistent with earlier data that found that PTH 1-34 treatment could increase calcium transport in vesicles created from human syncytiotrophoblast basement membranes[23] and with the intense expression of the PTH/PTHrP receptor in the intraplacental yolk sac.[21]

PTH 1-84 increased placental calcium transfer within 90 minutes of administration. At this early time point, it is likely that PTH acted directly through its receptor to open channels within the calcium-transporting cells of the placenta. Nevertheless, even at 90 minutes, we observed changes in the expression of mRNAs for VDR, vitamin D–binding protein, and several solute transporters. These changes in mRNA expression are unlikely to cause rapid enough changes in protein expression to account for the increased placental calcium transfer observed by 90 minutes, but the observed changes in these mRNAs indicate that PTH can regulate the expression of calciotropic genes and other solute transporters within the placenta. Moreover, the reduced placental expression of TRPV6, calbindin D-9K, VDR, and other solute transporters at baseline in *Pth* null fetuses is another indication that PTH may play a role in regulating placental function.

Although *Pth* null and *Gcm2* null fetuses were biochemically indistinguishable, the latter did have a low level of circulating PTH and a small increase in the apparent rate of placental calcium transfer. TRPV6, calbindin D-9K, and VDR each had significantly higher expression in *Gcm2* null compared with *Pth* null placentas, and this may in part explain why the rate of placental calcium transfer also differed between the two null genotypes. Moreover, PTH expression was detected in the placentas of WT and *Gcm2* null mice. Since the PTH/PTHrP receptor is intensely expressed in the placenta,[21] it is possible that PTH acts in a paracrine fashion to regulate placental genes and calcium transport. It is also conceivable that the small amount of plasma PTH in *Gcm2* nulls is derived from both thymic and placental sources.

In conclusion, we have determined that PTH contributes importantly to fetal calcium homeostasis because in its absence a fetal hypoparathyroid phenotype results with hypocalcemia, hypomagnesemia, hyperphosphatemia, low amniotic fluid mineral content, and reduced skeletal mineral content. PTH regulates the placental expression of genes involved calcium and other solute transfer and may contribute to the regulation of placental calcium transfer. PTH may contribute to placental gene expression and function both through both endocrine/systemic (parathyroid-derived) and paracrine (placental-derived) pathways. To our knowledge, no fetal or cord blood calcium measurements have been reported from human fetuses that lack parathyroids, such as owing to DiGeorge syndrome. Our results predict that DiGeorge syndrome will cause hypocalcemia in utero and impaired skeletal mineralization.

Disclosures

The authors state that they have no conflicts of interest.

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